



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>C12P 21/00, C12N 5/10, 15/09</b> <b>A01H 5/00, C07H 15/12</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/14831</b> <b>(43) International Publication Date:</b> 3 September 1992 (03.09.92)
<b>(21) International Application Number:</b> PCT/US92/01385 <b>(22) International Filing Date:</b> 21 February 1992 (21.02.92) <b>(30) Priority data:</b> 660,344 22 February 1991 (22.02.91) US 771,331 4 October 1991 (04.10.91) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 771,331 (CIP) Filed on 4 October 1992 (04.10.92) <b>(71) Applicant (for all designated States except US):</b> THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL AS- SOCIATES, INC. [US/US]; 505 Coast Boulevard South, La Jolla, CA 92037 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FITZMAURICE, Le- ona, Claire [US/US]; 10369 Caminito Surabaya, San Diego, CA 92131 (US). MIRKOV, Theodore, Erik [US/ US]; 12202 Creswick Court, San Diego, CA 92128 (US). ELLIOTT, Kathryn, Jane [US/US]; 3854 Baker Street, San Diego, CA 92117 (US). BUTLER, William, Owen [US/US]; 3737 Novel Drive #2109, San Diego, CA 92122 (US). KONNO, Yoshihiro [JP/JP]; 695-1 C-203, Johoji, Onishi, Tano, Gunma 370-14 (JP). DICKINSON, Craig, Duane [US/US]; 7987 Caminito Huerta, San Die- go, CA 92122 (US).		<b>(74) Agents:</b> WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US). <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (Eu- ropean patent), GN (OAPI patent), GR (European pa- tent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (Euro- pean patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US. <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NOVEL INVERTASE GENE(S) AND USES THEREOF		
<b>(57) Abstract</b>		
<p>Transgenic plants that are modified to produce fruits that have altered levels of soluble solids compared to non-transgenic plants of the same species are provided. The transgenic plants are prepared by introducing into plants DNA constructs that encode invertase operatively linked to DNA encoding regulatory regions that direct transcription of the DNA encoding invertase and operatively linked to DNA encoding amino acids that direct proper processing of the invertase through the secretory pathways of the plant and targeting of the invertase to the vacuole. In particular, DNA constructs encoding tomato plant vacuolar invertase in operative linkage with a developmentally regulated promoter region are provided. Preferred regulatory and structural DNA is obtained from genomic DNA clones and cDNA clones encoding tomato fruit vacuolar invertases from the commercial tomato plant, <i>Lycopersicon esculentum</i>, and wild tomato plant, <i>Lycopersicon pimpinellifolium</i>. Probes derived from the genomic DNA and cDNA, antibodies specific for tomato fruit invertase, and uses therefor, are also provided.</p>		

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**NOVEL INVERTASE GENE(S) AND USES THEREOF**

This application is a continuation-in-part of U.S. Patent Application Serial No. 07/771,331 to Fitzmaurice et al., filed October 4, 1991, "NOVEL INVERTASE GENE(S) AND USES THEREOF", which in turn is a continuation-in-  
5 part of U.S. Patent Application Serial No. 07/660,344 to Fitzmaurice et al., filed February 22, 1991, "NOVEL INVERTASE GENE(S) AND USES THEREOF". The subject matter of U.S. Patent Application Serial Nos. 07/771,331, and 07/660,344 are incorporated herein in their entirety by  
10 reference thereto.

**FIELD OF THE INVENTION**

The present invention is related to methods for improving the value of commercial varieties of plants by altering the phenotype of the plants and is related to  
15 the plants that exhibit the altered phenotype. In particular, this invention is related to transgenic tomato plants that have been genetically engineered to produce tomatoes that exhibit an altered solids content and an altered ratio of soluble solids to insoluble  
20 solids. Specifically, the solids content of the tomato fruit is altered by modifying the timing and level of expression of vacuolar invertase in ripening tomato fruit. This invention is also related to promoters and DNA for achieving such regulated expression in plants.

**BACKGROUND OF THE INVENTION**

25 Tomato solids include a water-soluble and a water-insoluble fraction. The insoluble solids in tomato fruit are primarily components of the cell wall and are responsible for the viscosity of processed tomato pulp.  
30 The water-soluble fraction contains the hexoses, glucose and fructose which constitute more than 90% of this fraction. Measurement of the content of these two sugars in a given fruit defines the "soluble solids content" of that fruit for commercial cultivars. The soluble solids  
35 content or ratio of soluble to insoluble solids is a major factor affecting the profitability of commercial

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tomato processing operations. The solids content is also important in determining the flavor and marketability of fresh market tomatoes.

The hexoses in ripened tomato fruit are produced by hydrolysis of sucrose, which is transported from the leaves, and by hydrolysis of accumulated starch, which is also derived from sucrose transported into the fruit, during fruit development. The enzyme which catalyzes the conversion of sucrose to the hexoses glucose and fructose, is beta-fructofuranosidase, commonly called invertase. Plants, including tomato, have at least two invertase activities, a soluble invertase located in the vacuole and an insoluble invertase activity bound to the cell wall.

There are characteristic differences in the activity of invertase and the distribution of sugars in plant tissues and in the fruit at different stages of ripeness. There are also differences in the activity profile of invertase and in the solids content among the fruits of different tomato species. For example, invertase activity increases in tomato fruit during ripening. Also, the fruit of *Lycopersicon pimpinellifolium*, which is a wild tomato species, is richer in invertase and expresses it earlier during ripening, and exhibits a higher soluble solids content than the cultivated tomato species, *Lycopersicon esculentum*.

Tomato growers and processors strive to develop tomato fruit that reflects the specific balance of soluble solids content and insoluble solids content desired for a particular tomato product. Traditionally, efforts to improve or alter this balance have focussed on the development of hybrid plants. For example, in an effort to increase the soluble solids content of cultivated tomatoes, such cultivated species have been crossed with wild tomato species that produce fruit with

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a higher soluble solids content than the cultivated varieties. The hybrid plants, however, not only acquire the desired trait but also tend to possess undesirable traits of the wild species.

5        There is a need, therefore, to produce improved versions of cultivated species of tomato, such as *L. esculentum*, that exhibit desirable traits of the wild species, such as a higher ratio of soluble solids to insoluble solids and a higher level of soluble solids,  
10 but that do not also have the undesirable traits of the wild species.

It would also be desirable to have the ability to produce cultivated plants that produce fruit that have a selected specific level of soluble solids content and  
15 ratio of soluble to insoluble solids content desired for a particular tomato product. In particular, it would be desirable to produce tomato fruit that have an increased soluble solids content as compared to that of presently available fruit and to thereby provide fruit that can be  
20 processed more economically.

Therefore, it is an object of this invention to provide transgenic tomato plants that express invertase earlier during ripening and express higher levels of invertase during fruit ripening than cultivated non-  
25 transgenic plants.

It is also an object of this invention to provide a means for regulating and altering the levels and ratios of soluble to insoluble solids in the fruit of cultivated tomato plants in order to select a specific level of  
30 soluble solids content and ratio of soluble to insoluble solids content desired for a particular tomato product.

It is also an object of this invention to provide tomato fruits that exhibit such soluble solids content and ratio of soluble to insoluble solids content.

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**SUMMARY OF THE INVENTION**

Transgenic tomato plants that have fruits with solids contents and ratios of soluble to insoluble solids that differ from non-transgenic plants of the same  
5 species are provided. In particular, transgenic tomato plants that produce fruits that have improved taste and processing properties are provided.

The altered soluble solids content and ratio of soluble to insoluble solids in tomato fruit are achieved  
10 by altering the timing of expression of an invertase and level of accumulation of such invertase in the vacuoles. The timing of expression of vacuolar invertase and the level of accumulation of vacuolar invertase in the plants are altered by increasing or decreasing expression of a  
15 gene or genes encoding invertase and by changing the time during the development of the plant, particularly the fruit, that one or more of such genes is expressed.

Methods for increasing the soluble solids content of tomato fruit produced by a tomato plant by introducing  
20 DNA constructs that contain DNA encoding an invertase are provided. The DNA construct encodes an invertase that is secreted and transported to the vacuoles or is modified so that the invertase is secreted and transported to the vacuoles.

25 In accordance with the methods, tomato plants are transformed with the constructs, and altered levels of invertase are expressed. In particular, DNA encoding the invertase is operatively linked to a promoter recognized by the plant RNA polymerase II. If the DNA encodes an  
30 invertase that is not a vacuolar invertase, DNA encoding the invertase is operatively linked to DNA that encodes vacuolar targeting sequences, and, if necessary, DNA encoding signal sequences.

Thus, a DNA construct encoding tomato fruit  
35 invertase under the control of a promoter that is

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functional in plants is introduced into cells of a tomato plant, the cells containing the construct are cultured under conditions that result in the development of transgenic tomato plantlets, and the plantlets are grown  
5 into tomato plants under conditions such that the DNA encoding tomato fruit invertase is expressed.

When recombinant tomato plants containing tomato fruit invertase under the transcriptional regulation of selected control sequences are grown, both the quantity  
10 and the timing of tomato fruit invertase production can be altered. The manner in which invertase expression is altered is a function of the regulatory sequences to which the invertase-encoding DNA is operably linked. The resulting transgenic plants produce fruit that has a  
15 soluble solids content and ratio of soluble solids to insoluble solids that differ from the non-transgenic plant.

DNA constructs made by fusing tomato invertase gene sequences with homologous or heterologous regulatory  
20 sequences are also provided. In preferred embodiments, the regulatory sequences, particularly the promoter region, are selected such that the onset of expression of recombinant tomato fruit invertase commences at an earlier stage of development of the tomato fruit than  
25 would otherwise occur when the same plant does not express the recombinant invertase. In particular, DNA encoding the invertase is operably linked to a developmentally regulated promoter selected so that the onset of expression of recombinant tomato fruit invertase  
30 begins at about the breaker stage of development of tomato fruit and continues until the tomato fruit has reached the red stage. The tomato fruit of a transgenic plant that contains this DNA construct should have a soluble solids content higher than the soluble solids  
35 content of tomato fruit produced by equivalent non-

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recombinant tomato plants. It is preferred that the transgenic fruit have a soluble solids content at least about 0.5% higher, preferably about 1% or more, than fruit of non-modified tomato plants.

5 DNA constructs including regulatory regions which contain fruit-specific developmentally controlled regulatory regions are provided. These regulatory regions include promoter regions that are effective for achieving regulated expression of heterologous DNA in  
10 transgenic plants.

The preferred promoter regions include, but are not limited to, constitutive promoters, such as the CaMV 35S promoter, and developmentally regulated promoters that confer fruit specificity and appropriate temporal control  
15 on the expression of the DNA encoding invertase. Such promoters include native *Lycopersicon* invertase promoters. DNA encoding regulatory regions upstream from the translation start codon of the structural invertase genes in the genomic clones from *L. esculentum* and *L.*  
20 *pimpinellifolium* and from other developmentally regulated genes are provided.

In addition, DNA encoding proteins and sequences that direct such proteins to the vacuoles are also provided. Such DNA encodes proteins that include signal  
25 sequences and specific C-terminal precursor peptide sequences, which target or sort proteins to the vacuole. DNA encoding such targeting and signal sequences may be operatively linked to DNA encoding an invertase that lacks such sequences. In preferred embodiments, such  
30 proteins include the tomato fruit invertase signal sequence, which includes amino acids 1-47 and 48-92 of Seq. ID No. 1, and an invertase carboxyl-terminal precursor or propeptide sequence, that includes residues 607-613 of Seq. ID No. 1, preferably included as part of  
35 the last 39 amino acids of tomato invertase (amino acids



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598-636 in Seq. ID No. 1) or a portion thereof that is sufficient to effect vacuolar targeting.

The constructs may be used to produce *L. esculentum* transgenic plants, or other transgenic plants, that  
5 express heterologous genes in a developmentally regulated manner. In particular, these constructs may be used to produce *L. esculentum* transgenic plants, or other transgenic plants, that express invertase under the control of the regulatory regions such that the levels of  
10 invertase expressed and the timing of expression of invertase differ from nontransgenic plants and the levels of soluble and insoluble solids in the transgenic tomato fruits differ from the fruits of nontransgenic plants.

In most preferred embodiments, DNA constructs  
15 containing the DNA encoding invertase from *L. esculentum* or *L. pimpinellifolium* operatively linked to DNA encoding the regulatory region of the invertase gene from *L. pimpinellifolium* or from other developmentally regulated genes that are expressed early during fruit  
20 ripening are introduced into a cultivated tomato species, such as *L. esculentum*, to produce transgenic plants that have an altered phenotype manifested as increased production of invertase earlier in fruit development as compared to that produced in the non-transgenic plants.  
25 Such transgenic tomato plants also can be used as a source for the production of substantially pure tomato fruit invertase and for the production of seeds that contain the heterologous DNA. Transgenic tomato plants in which the DNA encoding a mature invertase is operably  
30 linked to a secretion signal sequence, vacuolar targeting signals and to developmentally regulated promoter regions isolated from plants of the genus *Lycopersicon* are provided. Constructs including these signals and heterologous DNA for the purpose of producing transgenic  
35 tomato plants are also provided.

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In accordance with other embodiments, transgenic plants that express lower levels of soluble solids than the non-transgenic plant and methods for decreasing the soluble solids content of tomato fruit are provided.

- 5 Production of tomato fruits that have decreased soluble solids content is desirable when one seeks to obtain tomato fruit having a higher ratio of insoluble to soluble solids. Cultivars capable of producing fruit with a higher ratio of insoluble to soluble solids are of  
10 commercial value for the production of tomato products with high viscosity, such as tomato paste.

Transgenic plants that produce fruits that contain DNA constructs that result in decreased expression of invertase are provided. Reduced expression may be  
15 effected by methods such as cosuppression [for a discussion of cosuppression see Hooper, C. (1991) *J. NIH Res.* 3:49-54], by operatively linking a truncated form of a tomato fruit invertase gene to a promoter, or by expression of invertase antisense mRNA. Antisense RNA  
20 forms double-stranded RNA with the mRNA produced from the endogenous gene, thereby interfering with translation of the endogenous mRNA [see, e.g., Lichtenstein (1988) *Nature* 333:801-802]. To inhibit expression of the targeted gene, the antisense RNA can be less than full-  
25 length copy of the targeted mRNA [see, e.g., Grum et al. (1988) *Nuc. Acids Res.* 16: 4569-4581 and references cited therein].

In plants that express antisense invertase mRNA or truncated forms of the protein, the amount of invertase  
30 produced in the plant, particularly during fruit development, is substantially less than the amount of invertase produced when the plant does not express antisense invertase mRNA or a truncated form of invertase. The resulting fruit should have reduced  
35 levels of the hexoses. In preferred embodiments, such

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reduced levels in tomato fruit can be achieved by expressing, starting at the breaker stage and continuing through the ripe stage of fruit development, an antisense copy of part, or all, of the tomato fruit invertase mRNA or a truncated form of invertase mRNA in tomato fruit. As a result, reduced amounts of invertase are produced, and sucrose, which ordinarily would have been converted to glucose and fructose, may be converted into cell wall components.

10 In accordance with yet another embodiment, a tomato fruit produced by a transgenic tomato plant of the genus *Lycopersicon*, which is derived from a transgenic tomato plantlet which contains a recombinant construct encoding antisense tomato fruit invertase mRNA, such that the total soluble solids content and ratio of soluble to insoluble solids content of the fruit is altered by virtue of the decreased conversion of sucrose into the hexoses.

20 In accordance with a further embodiment, methods for identifying the presence of invertase-encoding nucleic acid sequences by contacting a sample containing RNA or single-stranded DNA with a probe containing all or a least a portion of the nucleic acid sequence set forth in Seq. ID Nos. 1, 2, or 4 are provided. Hybridizing DNA that encodes all or a portion of an invertase is isolated. In instances in which such hybridizing DNA encodes a portion of the invertase, it may be used to isolate full-length clones.

30 In preferred embodiments, isolated, substantially pure DNA encoding vacuolar invertases that have amino acid sequences substantially identical to the vacuolar invertases of the commercial tomato species, *L. esculentum*, and the wild tomato species *L. pimpinellifolium*, are provided. Genomic DNA and cDNA

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clones that encode the vacuolar invertase from each species are also provided.

Protoplasts containing the DNA constructs and seeds produced by the transgenic plants that include DNA that  
5 encodes the exogenous or heterologous invertase are also provided.

In accordance with still further embodiments, methods for determining the tomato fruit invertase content of a sample; methods for identifying the presence  
10 of invertase-encoding sequences in a cDNA expression library; methods for the recombinant production of tomato fruit invertase; methods for modulating the expression of tomato fruit invertase in solanaceous plant species; and methods for targeting protein product(s) expressed from  
15 heterologous genes by recombinant plants to the vacuoles are provided.

#### **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

##### **Definitions**

Unless defined otherwise, all technical and  
20 scientific terms used herein have the same meaning as is commonly understood by one of skill in the art. All publications mentioned herein are incorporated by reference thereto. All U.S. patents and publications cited herein are incorporated in their entirety by  
25 reference thereto.

As used herein, invertase refers to an enzyme that hydrolyzes sucrose to fructose and glucose and encompasses any protein that exhibits this activity in plants. The biological activity of invertase may be  
30 measured by one of several bioassays well-known in the art in which the sugars liberated by invertase activity are chemically quantified. Preferred invertases are those that, upon expression in a tomato plant, are transported through the processing pathway of the plant

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and targeted to the vacuoles. Tomato fruit vacuolar invertase is among those preferred herein.

As used herein, a precursor invertase refers to a protein that includes a leader or signal sequence that effects transport of the protein through plant processing pathways to yield mature protein and that includes a vacuolar targeting sequence to direct or sort the invertase to the vacuole. In the plant, signal sequences promote uptake of the protein into the endoplasmic reticulum (ER) of the plant cells.

As used herein, a signal or leader sequence, which expressions are used interchangeably, refers to a sequence of amino acids that directs transport of the translation product through the processing pathway of the host and results in the generation of a mature protein. The signal sequence includes or is modified to include one or a sequence of amino acids that is recognized by one or more host cell proteases. Such sequences may be interposed between the signal sequence and the protein, whereby, upon recognition of the processing site by the appropriate host cell protease, removal of the signal sequence may be effected. The signal sequence, processing sites and protein are referred to as a precursor protein, and the processed protein is referred to as the mature protein.

As used herein, regulatory sequences or signals also include sequences that are required for targeting proteins to selected plant organs, such as the vacuoles. Such sequences, vacuolar targeting sequences, present on the C-terminal end of the protein, effect transport of the protein to which they are linked to the vacuoles. If such sequence is absent and no other targeting sequence is present, the protein is directed to the default pathway and ultimately to the cell wall.

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The processing sequences, signal sequences and targeting sequences for use herein are those that are sufficient for directing mature invertase protein to which such sequences are linked to the vacuoles of the plant host in which the invertase is expressed. Any peptide or DNA encoding such peptide that effects proper processing and vacuolar targeting in plant hosts is contemplated for use herein. The preferred processing, signal, and targeting sequences for use herein are those that effect proper secretion, processing and targeting of the *L. esculentum* vacuolar invertase. These preferred signal sequences and targeting sequences include, but are not limited to, the vacuolar invertase signal sequence and carboxyl-terminal peptide. Other such sequences that are active in plants, such as the carboxyl-terminal propeptide (CTPP) of the barley lectin proprotein, the  $\beta$ -1,3-glucanase CTPPs of *Nicotiana tabacum* and *N. plumbaginifolia*, may also be used. The seven amino acids near the C-terminus of tomato fruit vacuolar invertase (amino acids 607 to 613 of Seq. ID No. 1) have 86% sequence similarity to a 7-residue region of the C-terminus of  $\beta$ -1,3-glucanase from *Nicotiana plumbaginifolia*, which is non-homologous to invertase in the rest of its sequence.

As used herein, precursor invertase refers to unprocessed invertase that includes sequences that direct the protein through the processing pathways of the plant. Such invertase includes the signal sequences and vacuolar targeting or sorting sequences.

As used herein, exogenous invertase refers to invertase that is encoded by DNA that is introduced into the plant and is expressed in the plant in addition to endogenous invertase. The exogenous invertase may be the same as the endogenous invertase. For example, in certain embodiments, the level of invertase expressed in

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the plant is altered by introducing a DNA construct that encodes a *Lycopersicon* invertase.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. Examples of heterologous DNA include, but are not limited to, DNA that encodes exogenous invertase and DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes.

As used herein, operative linkage of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences, refers to the functional relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame.

As used herein, isolated, substantially pure DNA refers to DNA fragments purified according to standard techniques employed by those skilled in the art, such as that found in Maniatis *et al.* [(1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY].

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression

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may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into  
5 cells for either expression of the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, expression vector includes vectors  
10 capable of expressing DNA fragments that are in operative linkage with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a  
15 phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells  
20 and/or prokaryotic cells and those that remain episomal or may integrate into the host cell genome.

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of DNA to which it is operatively linked. The promoter  
25 region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this  
30 recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. A developmentally



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regulated promoter is one that is turned on or off as a function of development.

As used herein, expression cassette refers to a DNA construct that includes DNA sequences that are functional for expression or encodes RNA or peptides functional for expression, and, if desired, processing and secretion of a mature protein in a selected host. Since such fragments are designed to be moved from vector to vector and into the host cell for both replication and expression, they are often referred to by those of skill in the art as "expression cassettes" or "cassettes". Accordingly an expression cassette includes DNA encoding a promoter region, a transcription terminator region, and sequences sufficient for translation, as well as any other regulatory signals, such as those that effect proper processing of the expressed protein or peptide.

As used herein, the term DNA construct embraces expression cassettes and includes DNA fragments that include more than one expression cassette.

As used herein, portions or fragments of the DNA constructs and expression cassettes are said to be operationally associated or operably or operatively linked when protein-encoding portions and regulatory regions are positioned such that expression, including transcription, translation and processing, of the protein-encoding regions is regulated by the DNA that encodes the regulatory regions.

As used herein, reference to "downstream" and "upstream" refers to location with respect to the direction of transcription from the promoter which regulates transcription of the invertase-encoding fragment.

As used herein, transgenic plants refer to plants containing heterologous or foreign DNA or plants in which the expression of a gene naturally present in the plant

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has been altered. Such DNA is said to be in operative linkage with plant biochemical regulatory signals and sequences. Expression may be constitutive or may be regulatable. The DNA may be integrated into a chromosome  
5 or integrated into an episomal element, such as the chloroplast, or may remain as an episomal element. In addition, any method for introduction of such DNA known to those of skill in the art may be employed.

As used herein, wild type plant refers to plants  
10 that are of the same species or are identical to the transgenic plants, but do not contain DNA or RNA that encodes the heterologous gene that may be expressed by the transgenic plant.

As used herein, homologous invertase refers to a  
15 protein that is sufficiently similar to tomato vacuolar invertase to catalyze the hydrolysis of sucrose to glucose and fructose and to so in the tomato plant.

As used herein, substantially homologous DNA refers to DNA that includes a sequence of nucleotides that is  
20 sufficiently similar to another such sequence to form stable hybrids under specified conditions. As used herein, substantially homologous DNA that encodes invertase includes DNA that hybridizes under conditions of low stringency to DNA that encodes an invertase and  
25 that encodes an invertase that functions as defined herein.

As used herein, a nucleic acid probe is a DNA or RNA fragment that includes a sufficient number of nucleotides to specifically hybridize to DNA or RNA that includes  
30 identical or closely related sequences of nucleotides. A probe may contain any number of nucleotides, from as few as about 10 and as many as hundreds of thousands of nucleotides. The conditions and protocols for such hybridization reactions are well known to those of skill  
35 in the art as are the effects of probe size, temperature,

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degree of mismatch, salt concentration and other parameters on the hybridization reaction. For example, two single-stranded nucleic acid segments have "substantially the same sequence," within the meaning of

5 the present specification, if (a) both form a base-paired duplex with the same segment, and (b) the melting temperatures of the two duplexes in a solution of 0.5 X SSPE differ by less than 10°C. If the segments being compared have the same number of bases, then to have

10 "substantially the same sequence", they will typically differ in their sequences at fewer than 1 base in 10.

As used herein, conditions under which DNA molecules form stable hybrids and are considered substantially homologous are such that the DNA molecules with at least

15 about 60% complementarity form stable hybrids. Such DNA fragments are herein considered to be "substantially homologous". In particular, DNA that encodes invertase is substantially homologous to another DNA fragment if the DNA forms stable hybrids such that the sequences of

20 the fragments are at least about 60% complementary and if a protein encoded by the DNA is invertase, i.e., catalyzes the conversion of sucrose into the hexoses, glucose and fructose. Thus, any nucleic acid molecule that hybridizes with nucleic acid that encodes all or

25 sufficient portion of invertase to be used as a probe, and that encodes invertase is contemplated for use in preparing DNA constructs and transgenic tomato plants as described herein.

As used herein, breaker stage refers to the stage in

30 fruit ripening at which the color of the fruit exhibits a definite break in color from green to tannish-yellow, pink or red, on not more than about 10% of the surface of the tomato fruit. When more than 10%, but less than about 30% of the fruit surface, in the aggregate, shows a

35 definite change in color from green to tannish-yellow,

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pink, red, or a combination thereof, the fruit is said to be at the "turning" stage. When more than 30%, but less than about 60% of the fruit surface, in the aggregate, is pink or red, the fruit is said to be at the "pink" stage, which is also the 3-inch intermediate stage, of development.

As used herein, all assays and procedures, such as hybridization reactions and antibody-antigen reactions, unless otherwise specified, are conducted under conditions recognized by those of skill in the art as standard conditions.

#### **Preparation of transgenic tomato plants.**

Transgenic tomato plants that express altered levels of invertase and produce fruits that exhibit altered solids content compared to non-transgenic plants are provided. The transgenic plants contemplated herein include those in which a heterologous or foreign gene encoding invertase, encoding an antisense invertase mRNA or encoding a truncated form of invertase has been inserted into the genome or into an episomal element. By virtue of the presence of the heterologous DNA, the plant is engineered to express a desired phenotype, including an altered soluble or insoluble solids content in the fruit, or to produce a protein, which can then be isolated upon harvesting the plant.

The preferred transgenic plants provided herein are transgenic tomato plants that express DNA encoding invertase under the control of either a constitutive or a developmentally regulated promoter region that is recognized by the tomato plant transcriptional machinery, including trans acting regulatory factors and RNA polymerase II, so that expression of the invertase is either constitutive or is developmentally regulated. In addition, the DNA introduced into the plant should include sequences that insure that the invertase that is

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expressed in the transgenic tomato plant is processed through the plant processing pathway that directs it to the vacuoles. Consequently, the DNA encoding the invertase must also encode the necessary regulatory sequences, including a signal sequence and vacuolar targeting sequence, to target the invertase to the vacuole. Such signals and targeting sequences may be isolated as part of the DNA encoding the invertase, if the invertase is a vacuolar invertase, or the DNA encoding the regulatory sequences may be operatively linked to the DNA that encodes the invertase.

The transgenic plants that contain and express invertase that is targeted to the vacuoles can be propagated and grown to produce fruit that exhibit an altered soluble solids content, altered insoluble solids content, or altered ratio of soluble to insoluble solids compared to the soluble and insoluble solids content of tomato fruit produced by unmodified tomato plants.

Transgenic tomato plantlets (*L. esculentum* cv. UC82) that contain DNA constructs encoding invertase in operative linkage with a promoter recognized by the plant RNA polymerase II have been regenerated in tissue culture. Such plantlets were produced by transformation of tomato with various DNA constructs prepared herein, including constructs in which the *L. esculentum* histidine decarboxylase (HDC) promoter, the *L. esculentum* invertase promoter, the *L. pimpinellifolium* invertase promoter, or the CaMV 35S promoter is fused to DNA encoding the *L. esculentum* tomato fruit vacuolar invertase.

The ratio of insoluble solids to soluble solids in tomato fruit may also be altered by operatively linking promoters to DNA encoding antisense or truncated forms of invertase. Expressing an antisense invertase mRNA or a truncated inactive form of invertase should result in

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reduced levels of the invertase gene product in the cell. Constructs that include truncated forms of invertase and that encode antisense invertase mRNA have been constructed. Such constructs have been introduced into  
5 tomato plants. These transgenic plants will be assayed for reduced expression of endogenous invertase.

**Preparation of DNA constructs that encode invertase.**

The DNA constructs containing DNA encoding invertase  
10 in operative linkage with regulatory sequences effective for expression and vacuolar targeting of the encoded invertase are prepared. These DNA constructs are alternatively referred to as recombinant DNA constructs, that is, fusions of various sequences, and may be  
15 produced using recombinant techniques well known in the art. The DNA constructs contain regulatory regions including promoters, transcription initiation sites, transcription termination sites, and, if necessary, vacuole sorting sequences, including signal sequences and  
20 carboxyl-terminal propeptides. Any or all of these component sequences may be homologous or heterologous to the host plant cell. Additional heterologous sequences may also be included if needed to facilitate transformation of the plant cell with the constructs or  
25 expression and proper processing and transport of the invertase.

The DNA constructs contain invertase-encoding sequences of nucleotides operably linked to genomic regulatory regions, including promoter regions. If the  
30 invertase encoded by the DNA is not directed to the vacuoles, DNA encoding appropriate regulatory sequences, such as the invertase signal and vacuolar targeting sequences, can be operably linked to the invertase coding DNA. Any sequence effective for such targeting may be  
35 used, such as, for example, the DNA encoding at least residues 607-613 of Seq. ID No. 1 and preferably residues

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598-636 of Seq. ID No. 1 or a portion thereof that includes residues 607-613 and is effective for vacuolar targeting or DNA encoding any sequence of amino acids known or shown to effect vacuolar targeting. Such sequences may be empirically identified or isolated from DNA that encodes proteins known to be directed to the vacuoles.

**Isolation of DNA encoding invertase.**

DNA encoding an invertase may be identified using the DNA or antiserum provided herein using any method known to those of skill in the art. DNA encoding any invertase that functions in a plant host, provided that it is operatively linked to sequences that effect vacuolar targeting, is contemplated for use herein.

DNA encoding invertase may be isolated by screening a library with all or a portion of DNA encoding tomato vacuolar invertase protein, which can be employed as a probe, for the identification and isolation of invertase-encoding sequences from an appropriate cDNA or genomic library or other sample containing DNA and RNA from plant and animal species. In particular, all, or a portion sufficient to identify related DNA, of the DNA encoding invertase provided herein is used a probe to isolate related DNA fragments.

Standard hybridization or other isolation techniques, as well known by those of skill in the art, can readily be employed for such purposes. Probes employed for such purpose typically have at least 14 nucleotides. Preferred probes employed for such purpose are those of at least about 50 nucleotides in length, and may include portions from the nucleotide sequence set forth in Seq. ID Nos. 1 or 4, or the various DNA molecules which encode the amino acid sequence set forth in Seq. ID No. 1; with nucleotide sequences of about 100 nucleotides or greater being especially preferred.

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Examples of such especially preferred sequences are those that have sequences set forth in Seq. ID No. 1, particular from the 5' coding region and the sequences encoding and surrounding what appears to be the active  
5 site of invertase, amino acid residues 295-307, particularly residues 298-306, of Seq. I.D. No. 1.

An exemplary 5' probe would be derived from the sequence of nucleotides 316-416 as set forth in Seq. ID No. 1; while an exemplary "active site" probe would be  
10 derived from the sequence of nucleotides 880-980 as set forth in Seq. ID No. 1. For ease of detection, such probes can be labeled with radioactive, chemiluminescent, or the like, labels.

The selected hybridizing DNA fragments may be  
15 characterized in order to ascertain whether they encode a full-length protein. If they do not, they may be used as probes to isolate full-length clones. The full-length clones may then be used to express the encoded protein, which may be assayed using standard assays for invertase  
20 activity, as defined herein. Selected full-length clones that encode invertase are then assessed for the presence of DNA encoding appropriate signal and vacuolar targeting sequences by any method known to the art, including producing transgenic plants and assaying for cloned  
25 invertase in the vacuole. If such signals are absent, the selected full-length clones may be modified by operatively linking such signals.

In addition, DNA encoding invertase that is not substantially homologous to tomato fruit invertase, such  
30 as yeast invertase, may be modified by the methods herein to produce DNA encoding an invertase that is properly sorted and targeted to the vacuoles. Such modified DNA is suitable for use herein.

The cDNA provided herein has been used to obtain  
35 genomic clones and also to obtain DNA encoding invertase



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from a related species. DNA encoding tomato fruit vacuolar invertase has been isolated by screening *L. esculentum* cDNA expression libraries with antisera raised against vacuolar invertase purified from *L. esculentum* fruit. The isolated cDNA was used to screen *L. esculentum* and *L. pimpinellifolium* genomic DNA libraries for invertase gene promoter sequences and *L. pimpinellifolium* cDNA libraries for DNA sequences encoding *L. pimpinellifolium* vacuolar invertase. In like manner the DNA and/or antiserum provided herein may be employed to isolate DNA encoding invertases from other sources.

DNA encoding tomato fruit vacuolar invertase has also been isolated herein using polyclonal antibodies that specifically bind to purified tomato fruit vacuolar invertase. These antibodies are specifically reactive with peptide sequences of tomato fruit invertase, but are substantially unreactive with other glycoproteins or glycan-containing groups. In addition, these antibodies can be employed in a variety of methods, including methods for determining the tomato fruit invertase content of a sample. Those of skill in the art can readily determine methodologies for using antibodies to measure the tomato fruit invertase content of a sample. See, for example, Clausen (1981) *Immunochemical Techniques for the Identification and Estimation of Macromolecules*, 2nd ed., Elsevier/North-Holland Biomedical Press, Amsterdam, the Netherlands.

The DNA encoding an invertase may also be isolated by screening a cDNA library with such antibodies in order to detect translation products of cDNA clones that encode all or a part of a vacuolar invertase or by screening a cDNA or genomic library with the DNA provided herein that encodes invertase. Use of these antibodies and DNA to identify cDNAs may be accomplished using methods known to

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those of skill in the art [see e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Vol. 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 12.1-12.44; Morton et al. (1987) *J. Biol. Chem.* 5 262: 11904-11907]. The antibodies have been used to screen a cDNA expression library and to identify DNA encoding tomato fruit vacuolar invertase. Expression libraries were prepared from poly(A)+ RNA isolated from the "pink" stage fruit of each tomato species. The 10 libraries were screened with the antibodies made against purified tomato fruit vacuolar invertase.

Selected clones, obtained using any screening method, may, if necessary, be used to obtain full-length clones. The clones may then be tested by any manner 15 known to those of skill in the art in order to ascertain whether the DNA encoding invertase includes sequences sufficient to direct it to the vacuoles. If such sequences are absent, DNA encoding targeting sequences may be operatively linked to the clone.

20 Any clone that encodes or that has been modified to encode a protein that has invertase activity as defined herein may be used for preparing DNA constructs and transferred into an appropriate host plant.

In particular, DNA encoding residues 1-636 of a 25 tomato fruit invertase preproprotein having the amino acid sequences set forth in Seq. ID No. 1 have been isolated. In addition, genomic DNA clones encoding both *L. esculentum* and *L. pimpinellifolium* invertase (Seq. ID Nos. 2 and 4, respectively) are provided.

30 Those of skill in the art recognize that, by virtue of the degeneracy of the genetic code, numerous DNA molecules have nucleic acid sequences that encode the amino acid sequence set forth in Seq. ID No. 1. For example, a presently preferred nucleic acid sequence is 35 set forth in Seq. ID No. 1, corresponding to the native

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nucleotide sequence encoding tomato fruit invertase from *L. esculentum*. Other sequences of nucleotides that encode this invertase or an invertase that functions equivalently may be obtained by methods known to those of skill in the art, including chemical synthesis and isolation of other invertase-encoding genes. Such invertases are limited to those that function in tomatoes and catalyze the hydrolysis of sucrose to fructose and glucose. If the invertase is not directed to the plant vacuole or is improperly processed in the tomato plant, DNA sequences encoding proper signal and vacuolar targeting sequences should be operatively linked to the invertase-encoding DNA.

Full-length cDNA and genomic clones that encode tomato fruit vacuolar invertase from both *L. esculentum* and *L. pimpinellifolium* have been isolated. The coding regions of both genes are identical. The promoter and other upstream regions of the genomic clones that encode the *L. pimpinellifolium* and *L. esculentum* invertases include repetitive regions. Each repeat unit includes the sequence 5'-TATTTAAT-3', which matches known plant nuclear protein binding sites. The *L. pimpinellifolium* repetitive region includes an additional repeat unit and differs at two other sites from the *L. esculentum* repetitive region.

Invertase gene expression in *L. esculentum* differs significantly from that in *L. pimpinellifolium*. For example, analysis of total RNA isolated from various stages of fruit development revealed that, in *L. pimpinellifolium*, invertase mRNA appears in green fruit, and is present at high levels in pink and red fruit. In *L. esculentum* cv. UC82, however, invertase mRNA does not appear until the pink stage of fruit development and is present at high levels only in red fruit. The apparent differences in fruit solids content may result from

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differences in gene expression, due to *cis*-acting factors, including the differences in nucleotide sequences of regulatory regions associated with the invertase genes, or regulatory factors acting in *trans*,  
5 such as factors which induce the earlier expression of the invertase gene in *L. pimpinellifolium*.

The promoter region from the *L. pimpinellifolium* invertase gene can be fused to DNA encoding invertase and introduced into *L. esculentum* tomato plants in order to  
10 produce *L. esculentum* plants in which invertase is expressed at an earlier stage in ripening than in the non-transgenic plants. Constructs in which the promoter region from the *L. pimpinellifolium* invertase gene is fused to the *L. esculentum* invertase gene have been  
15 prepared as means for altering expression of the *L. esculentum* invertase gene and to thereby increase the soluble solids content of the fruit.

Thus, the designed pattern of expression of the invertase gene in transgenic tomato plants may be  
20 accomplished by operatively linking it to a developmentally regulated promoter. DNA encoding developmentally regulated regulatory sequences obtained from the invertase gene and sequences that direct proper secretion and targeting of invertase have been identified  
25 and isolated, and DNA constructs containing DNA encoding invertase and fruit-specific genomic regulatory sequences are provided.

**Selection of developmentally regulated promoters and other regulatory sequences.**

30 **Identification and isolation of promoter regions.**

To accomplish the modification of invertase gene expression in tomato plants by transformation of tomato tissue with DNA encoding invertase, such DNA has been fused to developmentally responsive promoters. Preferred  
35 promoter regions and other regulatory sequences are those that are fruit specific and developmentally controlled.

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Such preferred regulatory regions include those that promote expression of recombinant invertase at an earlier stage of tomato fruit development than occurs when the subject plant does not express recombinant invertase.

- 5 Other embodiments include regulatory sequences that promote expression throughout fruit development.

Any developmentally regulated promoter region that, when linked to invertase-encoding DNA and introduced into a tomato plant host, does not promote expression until  
10 early in fruit ripening and promotes expression at high levels early during fruit ripening, is preferred for us herein. Especially preferred regulatory sequences are those which promote expression at about the breaker stage of tomato fruit development, the stage at which the fruit  
15 begins to turn pink or red, with continued promotion of expression until the tomato fruit has reached the red stage.

More specifically, regulatory regions have been isolated by screening a *L. pimpinellifolium* genomic DNA  
20 library with a probe containing cDNA encoding all or a portion of an invertase-encoding DNA sequence. A preferred subclone is one, as can be identified by restriction enzyme-mapping, that includes the 5' portion of an invertase-encoding sequence because there is a  
25 good chance that it will hybridize with the ATG start-site and upstream sequences of genomic clones.

For example, a 0.8-kb *XhoI-HindIII* 5'-end fragment of pTOM3-L1 was used as a cDNA probe and a plasmid containing an invertase-encoding fragment was isolated  
30 from an *L. pimpinellifolium* genomic DNA library. Such selected positive clones may be plaque-purified and restriction enzyme-mapped. Restriction enzyme-mapped clones having inserts extending the furthest upstream of the translation start site are then chosen for further  
35 characterization as the most likely to include the

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desired promoter sequences. For example, clone  $\lambda$ PI.6, which is a preferred clone described herein, include about 4 kb upstream of the translation start site.

Developmentally regulated promoter regions may also  
5 be isolated by any method known to those of skill in the art. For example, a method for isolating clones that encode a portion of a developmentally regulated gene is described in PCT Application WO 89/12230, which is based on U.S. Patent Application Serial No. 07/352,658 to  
10 Fitzmaurice et al., filed May 18, 1989, which is herein incorporated in its entirety by reference. The method provides a means to isolate promoter regions from genes that are, preferably, expressed in the tomato fruit prior to ripening, at the breaker stage. Use of this method is  
15 also described in the Examples herein. Any method by which developmentally regulated clones may be identified and isolated may be used.

The selected clones can be further characterized by northern analysis to select those that hybridize to mRNAs  
20 abundant at the developmental stage selected for study. For example, those that hybridize to mRNA that exhibits the desired developmentally regulated expression may be used as probes to screen genomic libraries in order to isolate the gene and regulatory sequences. The upstream  
25 portions can be sequenced and promoter regions identified and tested by fusing to reporter genes and looking for the appropriate regulation or pattern of expression in transgenic plants.

In particular, one such clone has been used to isolate  
30 a developmentally regulated promoter. This clone, which, upon expression *in vitro* yields a ~50 kDa translation product, that exhibits regulated expression during fruit ripening, has been used to screen an *L. esculentum* tomato genomic library and to isolate hybridizing clones.  
35 One such clone appears to encode a protein that has

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substantial homology with bacterial histidine decarboxylase and is herein referred to as the HDC gene. The portion of the clone upstream from the translation initiation site has been isolated and includes the  
5 promoter region, which appears to be a developmentally regulated promoter.

One such promoter region that has been selected, herein referred to as the *L. esculentum* HDC promoter region, is among those preferred for use herein. DNA  
10 fragments that include nucleotides from about 1 to about 888 or 889 of Seq. ID No. 3, or that are substantially homologous thereto and encode a developmentally regulated promoter are herein referred to as the HDC promoter. All or a portion of this region which promotes  
15 developmentally regulated expression is operatively linked to DNA encoding invertase. Constructs including this promoter region in operative linkage with DNA encoding invertase have been prepared. The constructs, HDC/3-L1.1, HDC/3-L1.2 and HDC/3-L1.3, contain different  
20 portions of the upstream sequences and are used to prepare transgenic plants. Such transgenic plants should express developmentally regulated levels of invertase.

In other preferred embodiments, invertase gene regulatory sequences from *L. esculentum* and  
25 *L. pimpinellifolium* are provided. These have been obtained by constructing genomic libraries of each species and screening them with a probe made from an invertase-encoding clone, such as plasmid pTOM3-L1, selected from a *L. esculentum* fruit cDNA library. The  
30 positive clones have been restriction enzyme-mapped and partially or completely sequenced. Thus characterized, the regulatory regions from these DNA fragments have been used to make fusions with invertase-encoding sequences. Thus *L. pimpinellifolium* promoter sequences can be  
35 fused to *L. esculentum* invertase-encoding regions.

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Other developmentally regulated promoters may be identified and isolated by means known to those of skill in the art. Such promoters preferably confer fruit specificity and an appropriate temporal control upon the expression of the coding sequences to which they are fused. For example, U.S. Patent No. 4,943,674 to Houck et al. describes methods and examples of developmentally regulated promoter regions, such as the 2A11 promoter.

Preferred promoter regions are fruit-specific developmentally regulated promoter regions, including, but not limited to, the promoter region from *L. pimpinellifolium* and *L. esculentum*, the HDC promoter, the polygalacturonase promoter, and the 2A11 gene. Most preferred promoter regions for use herein include the HDC promoter region (Seq. ID No. 3) and the regulatory regions from the *L. pimpinellifolium* genomic clone (Seq. ID No. 4).

Invertase gene promoter regions and other developmentally regulated promoter regions may also be linked to heterologous genes for developmentally regulated expression of genes of interest in plants. The regulatory regions, including the promoters, may be linked to other genes to achieve regulated expression of such genes in plants. For example, constructs have been prepared in which different portions of the HDC promoter region and the *Lycopersicon* invertase promoter regions have been fused to the coding region of the *E. coli*  $\beta$ -glucuronidase (GUS) gene.

Finally, invertase encoding DNA may be operatively linked to a constitutive promoter, such as the CaMV 35S promoter, and introduced into a plant. DNA constructs containing the CaMV 35S promoter have been constructed and used to prepare transgenic plants. By virtue of constitutive expression of the exogenous invertase in



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addition to expression of the endogenous invertase gene, invertase levels in the plant should increase. The resulting transgenic fruit should exhibit increased soluble solids content.

5           **Identification and isolation of DNA encoding processing and targeting signals.**

In addition to appropriate promoter selection, other regulatory sequences, including vacuolar targeting sequences must be included in the DNA construct in order  
10 to effect proper targeting of the heterologous invertase.

The identification and isolation of regulatory elements associated with tomato fruit vacuolar invertase genes can be accomplished by use of a cDNA clone encoding invertase as a probe. In addition, such sequences may  
15 be prepared synthetically and linked to DNA encoding an invertase that lacks such sequences.

DNA encoding sequences of amino acids that direct targeting or sorting of the invertase protein, as well as other proteins, to the tomato fruit vacuoles are  
20 provided. These include signal sequences, such as the invertase signal sequence, and carboxyl-terminal propeptide sequences. A 15 amino acid glycosylated carboxyl-terminal propeptide (CTPP) of the barley lectin proprotein is necessary for the efficient sorting of this  
25 protein to plant cell vacuoles [Bednarek et al. (1990) *The Plant Cell* 2:1145-1155]. In addition, it appears that the  $\beta$ -1,3-glucanase CTPPs of *Nicotiana tabacum* and *N. plumbaginifolia* may also be necessary for vacuolar sorting. Sequence comparison between the *Nicotiana*  $\beta$ -  
30 1,3-glucanase CTPPs and the carboxyl-terminal domain of the vacuolar tomato fruit invertase indicates 85% sequence similarity over a region of seven amino acids between residues 607 and 613 of tomato fruit invertase-encoding regions (see Seq. ID No. 1). Vacuolar  
35 targeting sequences, thus, may include DNA that encodes residues 607-613 of Seq. ID No. 1 and any additional

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portions of Seq. ID No. in that region necessary to effect vacuolar targeting.

DNA encoding tomato fruit invertase signal sequences and other sequences that are removed during processing are also provided. This region of the structural gene includes nucleotides encoding amino acids 1 through about 92 of the invertase-encoding Seq. ID No. 1. This DNA, as well as DNA identified as the carboxyl-terminal sequences (including residues 607-613 of Seq. ID No. 1) of the precursor protein described above, may also be used to direct the targeting of homologous or heterologous peptides into vacuoles by host recombinant solanaceous plants. Expression of the desired homologous or heterologous peptides from DNA constructs that include the above-described signal sequences and carboxyl-terminal coding sequences upstream of, and downstream of, respectively, and in reading frame with, the peptide, should direct a substantial portion of the expressed protein into the vacuoles of the host plant. Thus, invertase-encoding genes from sources other than tomato fruit, such as yeast, may be linked to DNA encoding the CTPP and DNA encoding the signal sequence from tomato invertase, thereby directing the gene product to the vacuole.

The DNA encoding invertase and constructs herein provided may also be introduced into a variety of hosts, such as solanaceous plants, prokaryotic or eukaryotic hosts, and invertase encoded by such DNA may be expressed and isolated. Exemplary hosts include yeast, fungi, mammalian cells, insect cells, and bacterial cells. The use of such hosts for the recombinant production of heterologous genes is well known in the art. In preferred embodiments, the DNA constructs are introduced into tomato plants and expressed by transgenic tomato plants during fruit development.

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**Introduction of heterologous DNA into plants.**

The DNA constructs provided herein are introduced into plants, plant tissues, or into plant protoplasts, particularly tomato plants, plant tissues, and  
5 protoplasts, to produce transgenic tomato plants.

Numerous methods for producing or developing transgenic plants are available to those of skill in the art. The method used is primarily a function of the species of plant. These methods include, but are not  
10 limited to, the use of vectors, such as the modified Ti plasmid system of *Agrobacterium tumefaciens*, the Ri plasmid system of *Agrobacterium rhizogenes* and the RNA virus vector, satellite tobacco mosaic virus (STMV). Other methods include direct transfer of DNA by processes  
15 such as PEG-induced DNA uptake, microinjection, electroporation, microprojectile bombardment, and direct and chemical-induced introduction of DNA [see, e.g., Uchimiya et al. (1989) *J. Biotech.* 12: 1-20 for a review of such procedures].

20 The resulting plants are grown, and fruits and seeds may be harvested. The transgenic plants may then be cross-bred in order to produce plants and seeds that are homozygous for the transgenic DNA. Such plants and seeds are contemplated for use herein.

25 The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

**EXAMPLE 1****CHARACTERIZATION OF INVERTASE PURIFIED FROM *L. ESCULENTUM* FRUIT.**  
30**A. Identification of a ~52 kDa protein as invertase.**

Gel analysis of samples of invertase purified from *L. esculentum* fruit by ammonium sulfate precipitation of the supernatant of a crude homogenate followed by DEAE-  
35 cellulose, Sephacryl S-200 and Concanavalin A-Sepharose column chromatography, revealed sequential enrichment of

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a major protein having an apparent molecular weight of ~52 kDa and two minor species of about 30 and 24 kDa.

Polyclonal antisera reactive against carrot cell wall invertase were used to identify the ~52 kDa protein isolated from *L. esculentum* fruit as invertase. The antisera, described by Lauriere et al. [(1988) *Biochimie* 70:1483-1491], also reacted with the ~30 and ~24 kDa proteins.

The predominant ~52 kDa protein present in purified preparations of *L. esculentum* fruit vacuolar invertase, as well as the two minor additional proteins (~30 and ~24 kDa) detected in these preparations, were subjected to N-terminal protein sequence analysis. The ~52 kDa protein and the ~24 kDa protein contain identical residues at the N-terminus (22 residues); the ~30 kDa protein yielded a 22-amino acid sequence that was different from the sequence of N-terminal residues obtained from the ~52 and ~24 kDa proteins. Subsequent analysis of the amino acid sequence deduced from a full-length invertase cDNA clone pTOM3-L1 (Example 2B) confirmed that the 22-residue sequence representing the N-terminus of the ~30 kDa protein is contained within the intact ~52 kDa protein.

Analysis of purified *L. esculentum* invertase showed that the ~52 kDa protein hydrolyzed sucrose to glucose and fructose and that it hydrolyzed raffinose to melibiose and fructose.

Invertase activity and substrate specificity were assayed by reacting 3.8 µg of protein obtained following Concanavalin A-Sepharose column chromatography with 90 mg/ml of substrate (sucrose or raffinose), in 13.6 mM citric acid and 26.4 mM NaHPO<sub>4</sub> (pH 4.8) at 30°C for 30 minutes. The reaction was stopped with the alkaline copper reagent of Somogyi [(1945) *J. Biol. Chem.* 160:51-68] and the liberated reducing sugars were measured according to Nelson [(1944) *J. Biol. Chem.*

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153:375-380]. To analyze substrate activity, the products of these reactions were subjected to thin layer paper chromatography using isobutanol:pyridine:H<sub>2</sub>O:acetic acid (12:6:4:1) as the solvent for ascending  
5 chromatography [Gordon et al. (1962) *J. Chromatog.* 8:44]. The positions of the carbohydrates were detected with alkaline silver nitrate [Chaplin (1986) "Monosaccharides", in *Carbohydrate Analysis, A Practical Approach*, Chaplin and Kennedy, eds; IRL Press,  
10 Washington, DC, pp. 1-36].

This characterization, in addition to the invertase activity assay results and the cross-reactivity to carrot invertase antibody, and protein sequence analysis confirmed identification of the ~52 kDa protein as *L. esculentum* invertase.  
15

**B. Identification of ~52 kDa protein as vacuolar invertase**

Invertase activities in protoplasts and vacuoles were evaluated and the purified invertase protein was  
20 compared to vacuolar proteins.

Protoplasts and vacuoles were purified from *L. esculentum* fruit tissue by squeezing two ripened tomato fruit into 35 ml of 25 mM Tris-MES [2-(N-morpholino)ethanesulfonic acid], pH 6.5, containing 0.7 M  
25 mannitol [Low pH Buffer; Boudet and Alibert (1987) *Methods in Enzymology* 148:74-81] and filtering the resulting suspension through two pieces of cheese cloth and stainless steel mesh (30 mesh). The filtrate was centrifuged at 100 x g for 3 min to collect protoplasts.  
30 The protoplasts were then resuspended in the same buffer and collected by centrifugation at 100 x g for 3 min.

Vacuolar fractionation was accomplished by a modification of the procedure described by Boudet and Alibert (1987) *Methods in Enzymology* 148:74-81. The  
35 protoplast fraction was diluted 1:4 with 20% (w/v) Ficoll in Low pH Buffer, then overlaid with 5 ml of Low pH

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Buffer containing 6 mg/ml DEAE-dextran and 10% (v/v) Ficoll, 2 ml of 6 mg/ml dextran sulfate (potassium salt) and 5% (v/v) Ficoll in 25 mM Tris-MES, pH 8.0, containing 0.7 M mannitol (High pH Buffer), and 2 ml of 1.2 mg/ml  
5 dextran sulfate and 1% (v/v) Ficoll in High pH Buffer followed by centrifugation at 2000 x g for 30 min. Vacuoles were recovered from the interface between the 5% and 1% Ficoll layers.

Protoplasts and vacuoles were lysed in the presence  
10 of invertase assay buffer and analyzed for invertase activity, as described above. The results of invertase assays of vacuolar and protoplast lysates revealed that the invertase activity in the vacuolar fraction was ~16-fold higher than the invertase activity in the  
15 protoplasts.

To determine if the vacuolar form of invertase was the form of invertase purified from *L. esculentum* fruit tissue, total protein from the purified vacuoles was subjected to SDS-PAGE and subsequent immunoblot analysis  
20 with the carrot invertase-specific antisera. The ~52 kDa, ~30 kDa, and ~24 kDa proteins detected in invertase purified from tomato fruit were detected in the vacuolar proteins.

**C. Production of polyclonal antisera to *L. Esculentum* vacuolar invertase.**  
25

Polyacrylamide gel-purified *L. esculentum* fruit vacuolar invertase (~52 kDa species obtained following separation on Concanavalin A-Sepharose) was excised from a gel, and 75 to 100 µg of protein were injected into  
30 rabbits, three times at intervals of two weeks, for the production of antibodies. The immunoglobulin fraction from immunized rabbits was subsequently purified from raw antiserum by Protein A-Sepharose affinity column chromatography. To remove antibodies reactive with  
35 glycans, the immunoglobulin fraction of this antiserum was passed over a horseradish peroxidase-Sepharose column

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which was prepared by coupling horseradish peroxidase to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ).

The antibodies reactive with tomato fruit invertase peptides did not bind to the column and the resulting "cleared" antibody fraction reacted specifically with tomato fruit invertase.

#### EXAMPLE 2

##### ISOLATION OF cDNA ENCODING *L. ESCULENTUM* VACUOLAR INVERTASE.

###### A. Library construction.

Total RNA was isolated from fresh *L. esculentum* cv. UC82 (grown from seeds obtained from Dr. Charles Rick, University of California at Davis, Dept. of Vegetable Crops) 3-inch intermediate fruit (i.e., fruit at the "turning" to "pink" stage of development) as described by De Vries et al. [(1988) In *Plant Molecular Biology Manual*, S.B. Gelvin, R.A. Schilperoot, and D.P.S. Verma, eds., Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. B6:1-13]. Poly(A)<sup>+</sup> RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (mRNA Purification Kit, Pharmacia LKB Biotechnology, Piscataway, NJ).

The poly(A)<sup>+</sup> RNA was used to construct size-selected cDNA libraries (of ~0.6 - 2 kb and ~2 - 4 kb and greater) in  $\lambda$ gt11. cDNA synthesis was carried out by the method of Lapeyre and Amalric [(1985) *Gene* 37:215-220] with the following modifications. A NotI-oligo(dT) primer-adaptor (Promega Corporation, Madison, WI) was used in first-strand synthesis. The addition of EcoRI adaptors was followed by digestion with NotI, generating cDNA inserts with a NotI site at the polyadenylated end and an EcoRI site at the opposite end. The cDNA was size-fractionated on a Sepharose CL-4B column. cDNAs of approximately 0.6 - 2 kb and 1.2 - 4 kb or greater in length were ligated into  $\lambda$ gt11 Sfi-Not (Promega Corporation, Madison, WI)

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which had been digested with *EcoRI* and *NotI*. The cDNA-containing  $\lambda$ gt11 vectors were then packaged and amplified (Gigapack<sup>R</sup> II Gold Packaging Kit, Stratagene Cloning Systems, La Jolla, CA).

5 **B. Library screening.**

To identify clones expressing tomato invertase, the immunological screening protocol described in Sambrook et al. [(1989) *Molecular Cloning: A Laboratory Manual*, Vol. 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 12.16-12.20] was used to screen directionally cloned *L. esculentum* fruit cDNA expression libraries. The primary antibody was antisera raised against tomato invertase protein and cleared of anti-glycan antibodies (see Example 1), and was pretreated as follows: 250  $\mu$ l each of *E. coli* strain Y1090 extract and crude  $\lambda$ gt11-*E. coli* strain Y1090 lysate were added to 5 ml of a 1:10 dilution of the tomato invertase antisera in the blocking buffer (described in the screening protocol), and the mixture was incubated 4 h at room temperature, then diluted 1:100 in blocking buffer. The secondary antibody was an anti-rabbit IgG-alkaline phosphatase (AP) conjugate (Promega Corporation, Madison, WI), diluted 1:7500 as described in the screening protocol.

25 Approximately 250 immunopositive plaques were detected in the primary screen of approximately 300,000 plaques. Six positive clones were plaque-purified by standard methods. The inserts of three of these clones, ranging from 1.1 to 1.5 kb in size, were subcloned into the *SfiI* and *NotI* sites of vector pGEM-11Zf(-) (Promega Corporation, Madison, WI). Plasmid mini-preps were performed according to the protocol of Sambrook et al. [(1989) *Molecular Cloning: A Laboratory Manual*, Vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p.1.40].



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The insert DNAs were sequenced according to the USB Sequenase<sup>R</sup> (United States Biochemical Corporation, Cleveland, Ohio) protocol. The complete sequence of the longest of the clones, pTOM3, contains 1339 bp which  
5 correspond to bases 840-2163 in Seq. ID No. 1, plus a poly(A) tail which has 15 adenine residues. Several classes of cDNA clones were identified, based on the location of the poly(A) tail.

Comparison of the deduced amino acid sequence of the  
10 insert in pTOM3 and the amino acid sequences of peptides generated by CNBr cleavage of the gel-purified preparation of *L. esculentum* vacuolar invertase revealed that a portion of the deduced amino acid sequence is present in one of the sequenced peptides.

15 The N-terminal protein sequence determined by sequencing the predominant ~52 kDa protein of partially purified preparations of *L. esculentum* fruit vacuolar invertase was not located in the pTOM3-deduced amino acid sequence, indicating that this cDNA clone does not encode  
20 a full-length invertase mRNA.

A 0.5 kb *Hind*III fragment of pTOM3, containing the 5' half of the DNA insert, was used as a probe to re-screen both the large insert (approximately 1.2 up to >4 kb) and small insert (~0.6-2.0 kb)  $\lambda$ gt11 *L. esculentum*  
25 cv. UC82 fruit cDNA expression libraries for full-length invertase cDNA clones, essentially according to the procedure of Maniatis et al. [(1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp.320-321, 326-328]. The  
30 filters were washed for 15 min each, once at 42°C in 2X SSC, 0.1% SDS, once at 42°C in 1X SSC, 0.1% SDS, once at 42°C in 0.5X SSC, 0.1% SDS, and once at 65°C in 0.1X SSC, 0.1% SDS.

Eleven hybridizing clones were plaque purified. The  
35 insert sizes of these clones ranged from ~1.4 to ~2.2 kb.

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Inserts from several of these clones were subcloned and sequenced as described above. The longest clone, pTOM3-L1, is 2199 bp in length (see, Seq. ID No. 1), encodes full-length tomato invertase, and also contains an  
5 additional 21 bp relative to pTOM3 preceding the poly (A) tail.

Comparison of the 3' ends of all of the *L. esculentum* cDNA clones sequenced revealed five classes of clones, based on the location of the poly(A) tail, at  
10 either the site indicated in Seq. ID No. 1, or a relative position of -23, -21, -17, or +13. All overlapping sequences were identical, except at a site 37 bp upstream of the beginning of the poly(A) tail in pTOM3-L1. That site contains a pyrimidine in all cases, C in 8 of 9  
15 clones and T in the other clone.

Comparison of the amino acid sequence deduced from pTOM3-L1 with the amino acid sequences of peptides generated by CNBr cleavage of vacuolar invertase purified from *L. esculentum* fruit and the amino-terminal sequence  
20 of purified invertase indicated that the protein encoded by this protein is invertase. In addition, sequence analysis of the ~30 kDa and ~24 kDa proteins which reacted strongly with anti-carrot invertase antisera in immunoblots of purified *L. esculentum* vacuolar invertase  
25 suggests that these proteins are degradation products of the mature invertase protein. The first 22 amino acids of the ~24 kDa protein were determined by sequence analysis to be identical to the first 22 amino acids of the ~52 kDa protein. The first 22 amino acids of the ~30  
30 kDa protein were determined by sequence analysis and did not correspond to the amino terminus of *L. esculentum* fruit vacuolar invertase. The first 22 amino acids of the ~30 kDa putative degradation product of tomato invertase are identical to amino acids 253-274 deduced  
35 from the nucleotide sequence of pTOM3-L1 (Seq. ID No. 1).

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The ATG translation start signal of pTOM3-L1 is the only in-frame ATG that results in an open reading frame from which a single peptide including all of the amino acid sequences derived from purified *L. esculentum* fruit vacuolar invertase can be deduced. Amino terminal sequence analysis of purified *L. esculentum* fruit vacuolar invertase indicates that the mature protein begins at the tyrosine residue at position 93 relative to the methionine encoded by the translation start codon (Seq. ID No. 1). Therefore, it appears that the first 92 amino acids of the protein encoded by pTOM3-L1 are co- or post-translationally cleaved, leaving a sequence of 544 amino acids extending from the amino terminus of the mature protein to the residue encoded by the codon preceding the stop codon.

Computer-assisted analysis of the resulting 544 amino acid peptide indicates that it has a molecular weight of ~60 kDa. The molecular weight of the mature deglycosylated tomato fruit vacuolar invertase was estimated to be ~45 kDa by SDS-PAGE. It is possible that additional post-translational modifications of the 636 amino acid precursor protein occur at the carboxyl terminus.

Based upon the assumption that the molecular weight of the mature protein is ~45 kDa, the carboxyl terminus of the mature protein has been predicted to be at amino acid position 502. This prediction is based upon the apparent molecular weight of the mature protein estimated by SDS-PAGE and thus is subject to experimental error of  $\pm 10$  amino acids.

### EXAMPLE 3

#### ISOLATION OF A cDNA ENCODING *L. PIMPINELLIFOLIUM* VACUOLAR INVERTASE.

A cDNA expression library was prepared from orange fruit of *L. pimpinellifolium* Trujillo, La Libertad Perú (grown from seeds obtained from Dr. Charles Rick,

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University of California at Davis, Dept. of Vegetable Crops) and was screened essentially as described in Example 2, except that the initial screen used <sup>32</sup>P-labeled *L. esculentum* cDNA clone pTOM3 as a probe. Five clones  
5 were identified, plaque purified, subcloned, and sequenced. The longest clone (pLP-19) contained an insert which is 30 bp shorter than the pTOM3-L1 insert at the 5' end and 7 bp longer at the 3' end prior to the poly(A) tail.

10 To isolate a full-length cDNA clone that encodes *L. pimpinellifolium* invertase, the *L. pimpinellifolium* fruit cDNA expression library was re-screened using a <sup>32</sup>P-labeled synthetic oligonucleotide complementary to nucleotides 7-33 of the *L. esculentum* cDNA (see Seq. ID  
15 NO. 1). Hybridization was carried out overnight at 42°C in 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS, 200 µg/ml denatured salmon sperm DNA and 10<sup>6</sup> cpm/ml radiolabeled probe. Several of the hybridizing clones were purified and characterized and compared with cDNA  
20 pTOM3-L1. *L. pimpinellifolium* clone pPIM11 was determined to be a full-length invertase-encoding cDNA that contains 7 nucleotides at the 5' end that are not present at the 5' end of pTOM3-L1 and 17 fewer nucleotides preceding the poly(A) tail than pTOM3-L1.  
25 Clone pPIM11 extends seven nucleotides farther in the 5' untranslated sequence direction than pTOM3-L1; the overlapping portions of the 5' ends of pPIM11 and pTOM3-L1 are identical.

The nucleotide sequences of the *L. pimpinellifolium*  
30 cDNA clones are essentially identical to those of the *L. esculentum* cDNA clones, differing only slightly at the 3' end. Several classes of *L. pimpinellifolium* cDNA clones with 3' ends of differing lengths were identified, as was the case for the *L. esculentum* clones. The *L.*  
35 *pimpinellifolium* cDNA poly(A) tails begin either at the

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same site as in *L. esculentum* clone pTOM3-L1 (see, Seq. ID No. 1) or at a relative position of -21, -17, or +7, and overlapping regions are identical in all clones. The site comparable to the variable site 37 bp upstream of the pTOM3-L1 poly(A) tail also contains a pyrimidine in all the *L. pimpinellifolium* cDNA clones, except that it is a T in 6 of the 7 clones sequenced and a C in only one clone. No other differences were found between the vacuolar invertase cDNAs of the two species.

10

**EXAMPLE 4****ISOLATION OF *L. PIMPINELLIFOLIUM* VACUOLAR INVERTASE GENE PROMOTER REGIONS.****A. Construction of a genomic library.**

Genomic DNA was isolated from seedling tissue of *L. pimpinellifolium* Trujillo, La Libertad Perú (grown from seeds obtained from Dr. Charles Rick, University of California at Davis, Dept. of Vegetable Crops) according to the procedure of Rogers and Bendich [(1988) *Plant Molecular Biology Manual*, pp. A6/1-10, Kluwer Academic Publishers, S. B. Gelvin, R. A. Schilperoot, eds.]. Restriction enzyme fragments generated by partial digestion with *Sau3AI* were cloned into  $\lambda$ FIX™ II (Stratagene, La Jolla, CA) according to manufacturer's instructions. The ligation reaction was packaged using Stratagene Gigapack™ II Gold packaging extracts.

25

**B. Library screening.**

A 0.8 kb *XhoI-HindIII* restriction enzyme fragment from plasmid pTOM3-L1 (a clone encoding invertase from the *L. esculentum* fruit cDNA library), see Example 2B, was labeled with  $^{32}\text{P}$ . This probe was used to screen the *L. pimpinellifolium* genomic library as described in Example 2, except that the wash in 0.5X SSC, 0.1% SDS was omitted. Two of the 12 positive clones,  $\lambda$ PI.1 and  $\lambda$ PI.3, were selected for further characterization.

35

The *L. pimpinellifolium* genomic library was then rescreened by the method described above with the  $^{32}\text{P}$ -

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labeled, gel-purified ~0.8 kb *XhoI-HindIII* fragment from the 5' end of the pTOM3-L1 insert. Six positive clones were selected for further characterization. Clone  $\lambda$ PI.6 was determined to encode the largest amount of sequence 5' from the initiation ATG.

### C. DNA sequencing.

Restriction enzyme fragments of the insert of  $\lambda$ PI.6 were subcloned and sequenced by the dideoxynucleotide chain termination method, using Sequenase<sup>R</sup> (United States Biochemical Corporation, Cleveland, Ohio). The sequenced region, provided in Seq. ID No. 4, includes the promoter and protein-encoding regions of the *L. pimpinellifolium* tomato vacuolar invertase gene.

The *L. pimpinellifolium* genomic sequence including the promoter and protein-encoding regions is set forth in Seq. ID No. 4. The transcription start site was identified by primer extension analysis by the method of Sambrook et al. [(1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., Vol. 1, pp. 7.79-7.83]. The oligonucleotide primer used in the primer extension reaction is complementary to nucleotides 74-107 of SEQ ID No. 1.

The transcription start site is located at nucleotide position 3668 of Seq. ID No. 4. The TATA box appears to be located at nucleotide positions 3637 through 3640. The translation start site appears to begin at nucleotide position 3686, and the stop codon begins at nucleotide position 7609. In addition, the cDNA 3' end sequences of different lengths share 100% sequence identity with the comparable regions of the genomic sequence. The variable site located near the 3' end of the cDNAs contains a T in the genomic clones sequenced.

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**EXAMPLE 5****ISOLATION OF *L. ESCULENTUM* INVERTASE GENE PROMOTER REGIONS.****A. Construction of genomic library.**

5       A genomic library was constructed in  $\lambda$  FIX<sup>TM</sup> II using DNA isolated from seedling tissue of *L. esculentum* cv. UC82 (grown from seeds obtained from Hunt-Wesson Foods, Inc., Pasadena, CA), according to the procedure described in Example 4.

10   **B. Library screening.**

      The insert of plasmid pTOM3 (see Example 2) was labeled with <sup>32</sup>P and used as a probe to screen the *L. esculentum* genomic library as described in Example 4. Four clones containing putative invertase-encoding  
15 sequences were identified, and three were selected for further characterization.

**C. DNA sequencing.**

      Restriction fragments of the insert of the above genomic clones were subcloned and sequenced. The  
20 sequenced regions, provided in Seq. ID No. 2, include the promoter and protein-encoding regions of *L. esculentum* tomato vacuolar invertase gene.

      The *L. esculentum* genomic sequence including the promoter and protein-encoding regions is set forth in  
25 Seq. ID No. 2. The transcription start site is located at nucleotide position 3502. The TATA box appears to be located at nucleotide positions 3471 through 3474. The translation start site appears to begin at nucleotide position 3520, and the stop codon begins at nucleotide  
30 position 7443. The cDNA 3' end sequences of different lengths share 100% identity with the comparable region of the genomic sequence. The variable site located near the 3' end of the cDNAs contains a C in the genomic clones sequenced.

35

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## EXAMPLE 6

**PREPARATION OF CONSTRUCTS CONTAINING TOMATO INVERTASE GENE PROMOTERS AND/OR CODING REGIONS.****5 A. *L. pimpinellifolium* invertase promoter/invertase gene constructs.**

Plasmid PI.6/BIN was constructed by inserting DNA containing regulatory and protein-encoding regions of the *L. pimpinellifolium* invertase gene into pBIN19 [Bevan (1984) Nucl. Acids Res. 12:8711-8721; Clontech, Palo Alto, CA.], a vector containing DNA sequences required for transferring DNA to plant cells. PI.6/BIN contains the *L. pimpinellifolium* invertase gene coding region as well as 3.7 kb of upstream and 3.4 kb of downstream sequence (nucleotides 1-10965 in Seq. ID No. 4).

15 Plasmid PI.6/BIN was constructed in two steps: (1) the ~7.8 kb insert DNA purified from plasmid pPI.6B7.8, which includes part of the *L. pimpinellifolium* invertase gene and ligated with BamHI-digested plasmid, pPI.6BgB2.9, which includes the remainder of the invertase gene, to yield a third plasmid; and (2) the ~10.7 kb insert, which includes nucleotides 1-10965 of Seq. ID No. 4 and 18 additional nucleotides (EcoRI, Sall, XhoI polylinker) at the 5' end, was purified from an EcoRI/BamHI (partial) digest of the plasmid and ligated  
20 with EcoRI- and BamHI-digested pBIN19 (Clontech, Palo Alto, CA) to yield PI.6/BIN.

**25 B. *L. esculentum* invertase promoter/invertase gene constructs.**

Plasmid pEI.23/BIN was constructed by inserting DNA containing regulatory and protein-encoding regions of the *L. esculentum* invertase gene into pBIN19. Plasmid pEI.23/BIN contains the *L. esculentum* invertase gene coding region as well as 3.5 kb of upstream and 3.4 kb of downstream sequences (nucleotides 1-10798 in Seq. ID No. 2).

35 2).



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Plasmid pEI.23/BIN was constructed in two steps: (1) a ~7.8 kb *Bam*HI insert was purified from a plasmid, pEI.3B7.8, which includes a portion of the the *L. esculentum* invertase gene and then ligated to a *Bam*HI-digested plasmid, pEI.2BB2, which contains the remainder of the gene, to produce a third plasmid, pEI.23BgB10.7, from which the ~10.7 kb insert was purified from an *Eco*RI/*Bam*HI (partial) digest of the plasmid; and (2) the purified fragment (nucleotides 1-10798 in Seq. ID No. 2 with the polylinker at the 5' end), was ligated to *Eco*RI- and *Bam*HI-digested pBIN19 to yield pEI.23/BIN.

**C. *L. esculentum* invertase promoter/GUS gene constructs.**

DNA from the promoter region of the *L. esculentum* gene between 3 base pairs and either 747, 913, or 1079 base pairs upstream from the initiator ATG from pEI.23BgB10.7 (Example 6B) corresponding to sequences between nucleotides 3517 and either 2773, 2607, and 2441 in Seq. ID No. 2 was amplified by the polymerase chain reaction (PCR) using Seq. ID No. 5 as a first oligonucleotide primer, which created an *Xba*I site 1 bp upstream of the initiator ATG, and Seq. ID No. 6 as a second primer (located in the region of direct repeats), which created a *Hind*III site 752 bp, 918 bp, 1084 bp and possibly additional sites upstream of the initiator ATG. These multiple priming sites are due to the presence of six 166-bp tandem direct repeats in this promoter.

The PCR products were digested with *Hind*III and *Xba*I and ligated with *Hind*III- and *Xba*I-digested pBI221 (Clontech, Palo Alto, CA) to fuse the promoter sequences with the GUS gene coding sequence in pBI221. Two correct plasmids, EI2GUS715 and EI2GUS1100, were identified by the production of ~750 bp and ~1100 bp fragments, respectively, upon digestion with *Hind*III and *Xba*I.

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Plasmid EI2GUS715 was digested with *EcoRI* and *HindIII*, and the 3.1 kb fragment was ligated with *EcoRI*- and *HindIII*-digested pBIN19 to produce EI2GUS715BIN.

The 3.4 kb *EcoRI*-*HindIII* fragment of plasmid  
5 EI2GUS1100 was ligated into pBIN19 by the same procedure used for EI2GUS715 to produce plasmid EI2GUS1100BIN.

#### EXAMPLE 7

##### TRANSFORMATION OF TOMATO PLANTS WITH INVERTASE PROMOTER CONSTRUCTS.

###### 10 A. Transformation of *L. esculentum* seedlings.

The transformation of seedlings of *L. esculentum* cv. UC82 (grown from seeds obtained from Ferry Morse Seed Co., Modesto, CA) was done essentially according to the protocol of Fillatti et al. [(1987) *Bio/Technology* 5:726-  
15 730]. Plasmids PI.6/BIN and EI.23/BIN (Example 6) were inserted into *Agrobacterium tumefaciens* strain LBA4404 [Clontech, Palo Alto, CA] through triparental mating [Ditta, G. (1986) *Meth. Enzymol.* 118] for transfer into *L. esculentum* seedlings.

20 The cultures were incubated at 27°C with 16 hours of light per day under 4,000 lux of light intensity. When kanamycin-resistant shoots reached a height of one inch, they were rooted on rooting medium, which is identical to regeneration 2Z medium except that it lacks hormones and  
25 contains 250 µg/ml cefotaxime and 50 µg/ml kanamycin. The transgenic shoots are grown into fruit-bearing transgenic tomato plants.

###### B. Assays for recombinant gene expression.

Tomato fruit tissues are assayed for invertase or  
30 GUS expression at various stages of fruit development. Invertase activity is determined according to the assay described in Example 1. GUS activity is determined essentially according to the protocol of Jefferson [(1987) *Plant Mol. Biol. Rep.* 5:387-405].

35 Protein concentration is determined according to the

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Protein Assay using reagents obtained from Bio-Rad Laboratories (Richmond, CA).

#### EXAMPLE 8

##### CONSTRUCTION OF HDC PROMOTER CONSTRUCTS

5

##### A. Isolation of a developmentally regulated gene.

##### 1. Construction of cDNA library.

Tomato fruit at the 3-inch intermediate stage was collected from greenhouse-grown *L. esculentum* cv. UC82 (grown from seeds obtained from Hunt-Wesson Foods, Fullerton, CA) and frozen in liquid nitrogen. Polysomes were prepared from 10 g of pulverized frozen tissue [Schröder et al. (1976) *Eur. J. Biochem.* 67:527-541] and RNA was extracted from the polysomes using an SDS-phenol-chloroform procedure similar to that described by Palmiter [(1974) *Biochemistry* 13:3606-3615] and stored at -70°C. Poly(A)+ RNA was selected by affinity chromatography on oligo(dT)-cellulose columns using the procedure of Aviv and Leder [(1972) *Proc. Natl. Acad. Sci. USA* 69:1408-1412], except that LiCl was used instead of NaCl.

A cDNA library was prepared by methods similar to those reported by Villa-Komaroff et al. [(1978) *Proc. Natl. Acad. Sci. USA* 75:3727-3731]. The cDNA molecules were made double-stranded with DNA polymerase I, Klenow fragment (New England BioLabs, Beverly, MA). To insure completion of the second strand synthesis, the DNA molecules were incubated with reverse transcriptase (Molecular Genetic Resources, Tampa, FL). The double-stranded molecules were made blunt-ended by digestion with S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN) and tailed with d(C) using terminal transferase (Ratliff Biochemicals, Los Alamos, NM).

The tailed DNAs were annealed to pBR322 DNA which had been digested at the PstI site and tailed with d(G) (New England Nuclear, Boston, MA). The recombinant

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plasmid DNA molecules were used to transform LE392 *E. coli* cells which were then plated on LB-tetracycline (15 µg/ml) plates. The resultant cDNA library was stored by the procedure of Hanahan and Meselson [(1980) *Gene* 10:63-67].

## 2. Library screening.

The cDNA library was screened to identify clones containing insert sequences which were expressed either constitutively or under developmental regulation. To achieve this, "early" and "late" stage RNA probes were prepared, labeled in a polynucleotide kinase reaction, and hybridized with the filter-bound DNAs.

### a. Preparation of probes.

Total RNA was prepared from 1-inch green "early" and 3-inch intermediate "late" *L. esculentum* cv. UC82 fruit and subjected to oligo-dT cellulose chromatography for the selection of poly(A)+ RNA essentially as described by Aviv and Leder [(1972) *Proc. Natl. Acad. Sci. USA* 69:1408-1412], but using LiCl for the binding instead of NaCl.

Poly(A)+ RNA prepared from early and late stages of *L. esculentum* cv. UC82 tomato fruit development was fractionated on a linear 5-20% sucrose gradient, and samples of RNA from gradient fractions were translated in an mRNA-dependent rabbit reticulocyte translation system by the method of Pelham and Jackson [(1976) *Eur. J. Biochem.* 67:247-256] to produce peptides labeled with L-(<sup>35</sup>S)-methionine [New England Nuclear (Boston, MA); October 1979 Manual]. Protein synthesis was assayed by determining the incorporation of TCA-precipitable label [Pelham and Jackson (1976) *Eur. J. Biochem.* 67:247-256], and the translation products were analyzed by electrophoresis on a 12.5% SDS acrylamide gel [Laemmli (1970) *Nature* 227:680-685] and fluorography.

### b. Library screening.

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Replica filters were prepared and the plasmids amplified [Hanahan and Meselson (1980) *Gene* 10:63-67] using 200 µg/ml chloramphenicol. DNA from cDNA clones was denatured, neutralized, and fixed to 150 mm

- 5 nitrocellulose filters [Maniatis et al. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York].

10 RNAs from a gradient fraction of one-inch green (early) RNA encoding proteins with a molecular mass of ~30 - ~60 kDa and from a similar gradient fraction of three-inch intermediate (late) RNA were labeled with <sup>32</sup>P in a polynucleotide kinase (Boehringer-Mannheim, Milwaukee, WI) reaction. These labeled fruit RNAs were then hybridized to approximately 10,000 cDNA clones (a  
15 fraction of the complete cDNA library) bound to nitrocellulose filters. Of 313 clones which yielded strong hybridization signals, 36% contained insert sequences which appeared to be expressed differentially at the two different stages of development.

- 20 **3. Identification of clone ptomUC82-3 as encoding a developmentally regulated protein.**

Plasmid DNA was prepared from clones which yielded strong hybridization signals, labeled with <sup>32</sup>P by nick translation and was used to probe northern blots of  
25 "early" and "late" fruit RNAs.

Total RNA was prepared from 1" green and 3" intermediate developmental stages of UC82 fruit as described above. RNA blots were prepared essentially as described by Thomas [(1980) *Proc. Natl. Acad. Sci. USA*  
30 77:5201-5205], and separate panels of RNA were hybridized with <sup>32</sup>P-labeled insert DNA from six cDNA clones. The autoradiographic patterns of hybridization indicated that clone ptomUC82-3 encodes a developmentally regulated, fruit-specific sequence which hybridized to a  
35 single RNA band with an apparent mobility of ~1.7 kb on a 1.5% agarose gel. These northern hybridization data, as

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well as hybridization/selection analyses, indicated that cDNA clone ptomUC82-3 corresponds to a gene which is expressed at low levels at early stages of fruit ripening, high levels at intermediate fruit ripening stages, and decreased levels in fully ripened fruit. The insert in ptomUC82-3, which was restriction-enzyme mapped and sequenced by the dideoxynucleotide chain termination method, was less than full-length cDNA clone but did contain an ATG start codon.

10 B. Isolation of the tomato HDC promoter.

A genomic library was constructed in  $\lambda$  FIX<sup>TM</sup>II (Stratagene, La Jolla, CA) using DNA isolated from seedling tissue of *L. esculentum* cv. UC82, as described in Example 4A. The genomic library was screened with a <sup>32</sup>P-labeled probe prepared from the 0.8 kb insert purified from cDNA ptomUC82-3 following digestion with *Pst*I. The screening conditions were identical to those described in Example 2B and clones which hybridized to the probe were identified and plaque-purified.

20 One of the clones isolated from the genomic DNA library,  $\lambda$ UC82-3.3, containing nucleic acids 1-4032 of Seq. ID No. 3, was shown by restriction enzyme mapping to contain putative regulatory regions upstream of the translation start site. A 3.7 kb *Sst*I-*Bgl*II fragment from the 5' end of this clone was subcloned. Sequence analysis of the insert of this subclone revealed that it contains six exons that have 95-100% identity with comparable positions of cDNA ptomUC82-3 and appears to include a promoter region. A fragment containing the remaining 347 nt upstream from the *Sst*I restriction site near the 5' end of the  $\lambda$ UC82-3.3 insert was subcloned and sequenced.

The results of a sequence similarity search through the GenBank database release 67.0 and EMBL database release 26.0 [Devereaux et al. (1984) *Nucl. Acids Res.*

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12:387-395] indicate a 60% similarity between the amino acid sequences predicted from cDNA clone ptomUC82-3 and the *Morganella morganii* bacterial histidine decarboxylase gene.

- 5       The promoter-containing region of  $\lambda$ UC82-3.3, nucleotides 1-888 of Seq. ID No. 3, is herein referred to as the HDC promoter.

**C. HDC promoter/tomato fruit invertase constructs**

**1. HDC/3-L1.1.**

- 10       Construct HDC/3-L1.1 contains 538 bp of the HDC promoter region from  $\lambda$ UC82-3.3 (nucleotides 349 to 886 of Seq. ID No. 3) fused to the *L. esculentum* cv. UC82 invertase cDNA pTOM3-L1 insert (nucleotides 1 to 2199 of Seq. ID No. 1), which is fused at the 3' end to the NOS  
15 (nopaline synthase) terminator.

pTOM3-L1 was digested with *Xho*I, made blunt-ended with T4 DNA polymerase, then digested with *Not*I to yield a 2202 bp fragment containing 3 nucleotides from the vector polylinker (AGC) plus the complete *L. esculentum*  
20 cv. UC82 invertase cDNA coding sequence (nucleotides 1 to 2199 of Seq. ID No. 1).

The above fragment from pTOM3-L1, the fragment containing the HDC promoter (nucleotides 349 to 886 of Seq. ID No. 3) were purified and ligated with *Not*I-,  
25 *Sst*I-digested pGEM-11Zf(-) (Promega Corporation, Madison, WI). The resulting plasmid was called -540/3-L1.

The NOS terminator is contained in plasmid pBI101 (Clontech, Palo Alto, CA). Plasmid pBI101 was digested with *Sst*I and *Hind* III and made blunt-ended with T4 DNA  
30 polymerase yielding an ~10 kb vector fragment. The purified vector fragment was ligated to the DNA insert of -540/3-L1, which had been prepared by digestion with *Not*I and *Sst*I and made blunt-ended with T4 DNA polymerase, to produce construct HDC/3L-1.1.

- 35       **2. HDC/3-L1.2.**

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Construct HDC/3-L1.2 is identical to HDC/3-L1.1 except that it contains 886 bp of the HDC promoter region from  $\lambda$ UC82-3.3 rather than 538 bp. Construct HDC/3-L1.2, thus, contains 886 bp of the HDC promoter region from  
5  $\lambda$ UC82-3.3 (nucleotides 1 to 886 of Seq. ID No. 3) fused to the tomato invertase gene (nucleotides 1 to 2199 of Seq. ID No. 1), which is fused at the 3' end to the NOS (nopaline synthase).

### 3. HDC/3-L1.3.

10 Construct HDC/3-L1.3 is identical to HDC/3-L1.1 except that it contains 690 bp of the HDC promoter region from  $\lambda$ UC82-3.3 rather than 538 bp. Construct HDC/3-L1.3, thus, contains 690 bp of the HDC promoter region from  
15  $\lambda$ UC82-3.3 (nucleotides 1 to 690 of Seq. ID No. 3) fused to the *L. esculentum* cv. UC82 invertase cDNA (nucleotides 1 to 2199 of Seq. ID No. 1) which is fused at the 3' end to the NOS (nopaline synthase) terminator.

## D. HDC-promoter/GUS constructs.

### 1. HDC/GUS.1.

20 Construct HDC/GUS.1 contains a promoter fragment from  $\lambda$ UC82-3.3 which extends from 794 to 3 bp upstream of the ATG start codon (nucleotides 94 to 886 in Seq. ID No. 3) fused to the *E. coli* GUS gene.

Plasmid pUC82-3.3NH was digested with *DdeI*, the ends  
25 of the resultant fragment were filled in with Klenow DNA polymerase, and the 792 bp fragment was isolated and purified. Plasmid pUC82-3.3NH was constructed by inserting the 3.4 kb restriction enzyme fragment, which extends from the *NotI* site in the vector polylinker to  
30 the first *HindIII* site from the 5' end of the  $\lambda$ UC82-3.3 insert, into the *NotI* and *HindIII* sites of pGEM-11Zf(-) (Promega Corporation, Madison, WI) to produce pUC82-3.3NH.

Plasmid pBI101.3/pUC was made by inserting the 2200  
35 bp *EcoRI-HindIII* fragment of pBI101.3 (Clontech, Palo



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Alto, CA) into *EcoRI-HindIII*-digested pUC119 [Vieira and  
Messing (1987) In *Methods in Enzymology*, R. Wu and L.  
Grossman, Eds., Vol. 153, pp. 3-11, Academic Press, New  
York]. The 792 bp fragment was ligated to pBI101.3/pUC  
5 which had been digested with *HindIII* and *BamHI*, and the  
resulting plasmid was called -790/GUS.

The 3 kb *EcoRI-HindIII* fragment containing the HDC  
promoter-GUS fusion was isolated from -790/GUS and  
ligated to *EcoRI*- and *HindIII*-digested pBIN19 to produce  
10 HDC/GUS.1.

## 2. HDC/GUS.2

Construct HDC/GUS.2 contains 690 bp of the HDC  
promoter region from  $\lambda$ UC82-3.3 (nucleotides 1 to 690 of  
Seq. ID No. 3) fused to the *E. coli* GUS gene.

15 Plasmid pUC82-3.3NH was digested with *XbaI* and *SspI*,  
and the 710 bp fragment was isolated and purified. The  
710 bp fragment was ligated to purified *XbaI*- and *SmaI*-  
digested pBI101.3/pUC to create -690/GUS.

The 2.9 kb *EcoRI-HindIII* fragment containing the HDC  
20 promoter-GUS fusion was isolated from -690/GUS and  
ligated to *EcoRI*- and *HindIII*-digested pBIN19 to produce  
HDC/GUS.2.

## EXAMPLE 9

### 25 TRANSFORMATION OF *L. ESCULENTUM* WITH HDC PROMOTER CONSTRUCTS AND ANALYSIS FOR RECOMBINANT GENE EXPRESSION.

The transformation of seedlings grown from *L.*  
*esculentum* cv. UC82 seeds was performed essentially by  
the protocol of Fillatti et al. [(1987) *Bio/Technology*  
5:726-730], as described in Example 7.

30 Invertase and GUS expression in the fruit of  
transformed tomato plants may be assayed as described in  
Example 7.

## EXAMPLE 10

### INVERTASE C-TERMINAL/GUS CONSTRUCTS

35 Two constructs have been assembled using the signal  
and targeting sequences from secreted proteins. The

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first of these constructs (35S/GUS44) was assembled to express a fusion protein with the signal sequence from phytohemagglutinin-L (PHA) fused to the amino-terminus of *E. coli* GUS and was designed to allow GUS to be targeted to the endoplasmic reticulum and then secreted. The second construct (35S/GUS-INV) incorporates the C-terminus of tomato fruit vacuolar invertase into GUS and should target GUS to the vacuole.

35S/GUS44 was constructed from plasmid pA35/PHIN44 [Dickinson et al. (1991) *Plant Physiol.* 95:420-525]. The yeast invertase coding sequence in pA35/PHIN44 was replaced by the GUS coding sequence of plasmid pGUSN358-S (Clontech, Palo Alto, CA) by digesting pA35/PHIN44 with *Sph*I and ligating this vector with a *Sph*I-digested fragment derived from PCR amplification of plasmid pGUSN358-S, using Seq. ID No. 7, which anneals to the 5' end of the GUS gene coding sequence, and Seq. ID No. 8, which anneals to the 3' end of the GUS gene coding sequence, as primers.

The 1.8 kb fragment obtained after PCR amplification of pGUSN358-S was digested with *Sph*I and ligated into pA35/PHIN44 to produce plasmid 35S/GUS44. This plasmid contains the following noteworthy features: 1) a cauliflower mosaic virus promoter, CaMV 35S, for high-level expression; 2) the coding sequence for the first 44 amino acids of PHA-L which includes the 20 amino acid signal sequence for efficient translocation across the ER membrane; 3) the GUS reporter protein coding sequence fused in-frame with the PHA sequence and modified by deletion of a glycosylation site that allows GUS to move through secretory system; 4) a unique *Pst*I restriction site which immediately precedes the termination codon of GUS for in-frame C-terminal fusions; and 5) an octopine synthase transcriptional terminator. Upon introduction of this construct into a plant, active

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GUS which is secreted by the default pathway to the plant cell wall should be produced.

To demonstrate the ability of the C-terminus of tomato invertase to target heterologous proteins to the vacuole, the coding sequences for the last 39 amino acids of tomato invertase (amino acids 598-636 in Seq. ID No. 1) were fused in-frame to the 3' end of the GUS gene contained in plasmid 35S/GUS44.

First, plasmid 35S/3L-1b was constructed to place the OCS transcriptional terminator after the invertase cDNA sequence and to thereby facilitate subsequent steps. Plasmid pTOM3-L1 was digested with *NotI*, filled in with Klenow DNA polymerase, digested with *XhoI*, and the 2.2 kb fragment was purified and cloned into the CaMV 35S promoter/OCS terminator vector pA35. Plasmid pA35 was prepared for this cloning by digesting with *SphI*, filling-in with Klenow DNA polymerase, and then digesting with *SalI*. The resulting clone was named 35S/3L-1b and was used for PCR amplification of the 3' end of the invertase sequence.

For amplification of a fragment containing the 3' end of the 35S/3L-1b insert, a synthetic oligonucleotide was designed which included a new *PstI* restriction site to facilitate the in-frame fusion of GUS and invertase sequences. The sequence of one primer was Seq. ID No. 9. The second primer was the pUC/M13 reverse primer (New England Biolabs, Inc., Beverly, MA). Amplification by PCR was conducted according to the procedure of Perkin-Elmer/Cetus (Norwalk, CT). The resultant 600 bp fragment was then digested with *PstI* and *HindIII* and ligated into 35S/GUS44, which had been digested with *PstI* and *HindIII* and purified, to produce plasmid 35S/GUS-INV.

These constructs are transformed into tomato tissue by methods described in Example 7. The resulting

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transformed plants are then assayed for GUS activity as described in Example 7.

#### EXAMPLE 11

##### **CaMV 35S PROMOTER/TOMATO FRUIT INVERTASE CONSTRUCTS**

##### **5 A. 35S/3-L1/BIN Overproducing construct.**

##### **1. Construction.**

The cDNA sequence encoding tomato fruit vacuolar invertase (nucleotides 1-2199 of Seq. ID No. 1) was inserted between the CaMV 35S promoter and the nopaline synthase (NOS) terminator in vector pCAMVCN (Pharmacia LKB Biotechnology, Piscataway, NJ). Plasmid pCAMVCN was digested with *Pst*I, blunt-ended with T4 DNA polymerase, purified, and ligated with the purified 2202 bp *Xho*I-*Not*I fragment of pTOM3-LI, which was also made blunt-ended  
15 with T4 polymerase. The resulting clone, named 35S/3-L1, was ligated into pBIN19 as a cassette fragment containing the CaMV 35S promoter, the invertase cDNA sequence, and the NOS terminator, to produce 35S/3-L1/BIN. This subcloning was performed by digesting 35S/3-L1 at the 3'  
20 end with *Bgl*II and at the 5' end with a partial *Xba*I digestion. The 3.0 kb fragment was purified and ligated into pBIN19 prepared by digestion with *Xba*I and *Bam*HI.

##### **2. Transformation and expression.**

Seedlings grown from seeds of *L. esculentum* cv. UC82  
25 were transformed with 35S/3-L1/BIN essentially by the protocol of Fillatti et al. [(1987) *Bio/Technology* 5:726-730], as described in Example 7. To determine the level of invertase activity in plants generated from the transformed seedlings, mature leaf tissue from the  
30 transgenic plants and control non-transgenic plants that had been growing in soil for two months was assayed as follows. Tissue samples (1 g) were homogenized in a mortar and pestle with 2 ml homogenization buffer (0.25 M Tris phosphate, pH 7.6, containing 1 mM EDTA and 5 mM  
35 DTT). All steps were performed at 4°C. Homogenates were

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centrifuged for 10 min at 14,000 x g and the supernatant was stored on ice. Leaf extracts were adjusted to contain equal concentrations of protein, which were determined by the Bradford Reagent method (BioRad Laboratories, Richmond, CA). Samples of the leaf extracts were analyzed in invertase activity gels, which were performed according to the method of Gabriel and Wang [(1969) *Anal. Biochem.* 27:545-554], as modified by Carlson et al. [(1981) *Genetics* 98:25-40]. Purified tomato fruit invertase was used as a positive control in these assays.

Tomato plants transformed with 35S/3-L1/BIN were shown to contain high levels of invertase activity in their leaves. With the conditions used for the extraction and assay, no invertase activity was detected in extracts from non-transgenic tomato leaves.

**B. Antisense construct 35/3-L1(-).**

An antisense invertase construct designed to reduce expression of vacuolar invertase in tomato fruit has been prepared. The vacuolar tomato fruit invertase clone pTOM3-L1 cDNA insert (nucleotides 1-2199 of Seq. ID No. 1) was inserted into the CaMV 35S promoter/terminator cassette in the reverse orientation to create 35S/3-L1(-). pTOM3-L1 was digested with NotI, blunt-ended, digested with XhoI, and the 2202 bp fragment was purified and cloned into pA35 prepared by digestion with SmaI and SalI. 35S/3-L1(-) contains the CaMV 35S promoter fused to an antisense pTOM3-L1 cDNA and the OCS transcriptional terminator. This fusion construct was ligated as an EcoRI-SstI fragment into the corresponding sites of pBIN19 to yield plasmid 35S/3-L1(-)BIN.

**C. Cosuppression construct 35S/3-L1(P).**

A construct for use in cosuppression of endogenous invertase expression was constructed by removing a coding segment from 35S/3-L1 to create a construct 35S/3-L1(P)

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which encodes a truncated, nonfunctional protein. To prepare construct 35S/3-L1(P), 35S/3-L1 was digested with *Pst*I, which digests at two sites (nucleotides 1205 and 1386 in Seq. ID No. 1) within the invertase coding  
5 region, and religated. This produced a 181 bp deletion, creating a shift in the reading frame after codon 400 in Seq. ID No. 1 and the introduction of a stop codon four codons downstream.

Using the same strategy as described above for  
10 35S/3-L1/BIN, 35S/3-L1(P) was mobilized into pBIN19 as an *Xba*I fragment (obtained following a partial digestion) to create 35S/3-L1(P)BIN.

Transgenic plants that contain this construct should express reduced levels of invertase compared to a non-  
15 transgenic plant of the same species.

Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

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## SEQUENCE LISTING

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(ii) TITLE OF INVENTION: NOVEL INVERTASE GENE(S) AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 9

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/660,344  
(B) FILING DATE: 22-FEB-1991

## (viii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/771,331  
(B) FILING DATE: 04-OCT-1991

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## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619-552-1311

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2199 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 7..1917  
(D) OTHER INFORMATION: /product= "L. esculentum vacuolar invertase"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTATT ATG GCC ACT CAG TGT TAT GAC CCC GAA AAC TCC GCC TCT CGT	48
Met Ala Thr Gln Cys Tyr Asp Pro Glu Asn Ser Ala Ser Arg	
1 5 10	
TAC ACA TTA CTC CCG GAT CAA CCC GAT TCC GGC CAC CGG AAG TCC CTT	96
Tyr Thr Leu Leu Pro Asp Gln Pro Asp Ser Gly His Arg Lys Ser Leu	
15 20 25 30	

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AAA ATC ATC TCC GGC ATT TTC CTC TCC GTT TTC CTT TTG CTT TCT GTA Lys Ile Ile Ser Gly Ile Phe Leu Ser Val Phe Leu Leu Leu Ser Val 40 45	144	35
GCC TTC TTT CCG ATC CTC AAC AAC CAG TCA CCG GAC TTG CAA ATC GAC Ala Phe Phe Pro Ile Leu Asn Asn Gln Ser Pro Asp Leu Gln Ile Asp 50 55 60	192	
TCC CGT TCG CCG GCG CCG CCG TCA AGA GGT GTT TCT CAG GGA GTC TCC Ser Arg Ser Pro Ala Pro Pro Ser Arg Gly Val Ser Gln Gly Val Ser 65 70 75	240	
GAT AAA ACT TTT CGA GAT GTA GCC GGT GCT AGT CAC GTT TCT TAT GCG Asp Lys Thr Phe Arg Asp Val Ala Gly Ala Ser His Val Ser Tyr Ala 80 85 90	288	
TGG TCC AAT GCT ATG CTT AGC TGG CAA AGA ACG GCT TAC CAT TTT CAA Trp Ser Asn Ala Met Leu Ser Trp Gln Arg Thr Ala Tyr His Phe Gln 95 100 105 110	336	
CCT CAA AAA AAT TGG ATG AAC GAT CCT AAT GGA CCA TTG TAT CAC AAG Pro Gln Lys Asn Trp Met Asn Asp Pro Asn Gly Pro Leu Tyr His Lys 115 120 125	384	
GGA TGG TAC CAC CTT TTT TAT CAA TAC AAT CCA GAT TCA GCT ATT TGG Gly Trp Tyr His Leu Phe Tyr Gln Tyr Asn Pro Asp Ser Ala Ile Trp 130 135 140	432	
GGA AAT ATC ACA TGG GGC CAT GCT GTA TCC AAG GAC TTG ATC CAC TGG Gly Asn Ile Thr Trp Gly His Ala Val Ser Lys Asp Leu Ile His Trp 145 150 155	480	
CTC TAC TTG CCT TTT GCC ATG GTT CCT GAT CAA TGG TAT GAT ATT AAC Leu Tyr Leu Pro Phe Ala Met Val Pro Asp Gln Trp Tyr Asp Ile Asn 160 165 170	528	
GGT GTC TGG ACA GGG TCC GCT ACC ATC CTA CCC GAT GGT CAG ATC ATG Gly Val Trp Thr Gly Ser Ala Thr Ile Leu Pro Asp Gly Gln Ile Met 175 180 185 190	576	
ATG CTT TAT ACC GGT GAC ACT GAT GAT TAT GTG CAA GTG CAA AAT CTT Met Leu Tyr Thr Gly Asp Thr Asp Asp Tyr Val Gln Val Gln Asn Leu 195 200 205	624	
GCG TAC CCC GCC AAC TTA TCT GAT CCT CTC CTT CTA GAC TGG GTC AAG Ala Tyr Pro Ala Asn Leu Ser Asp Pro Leu Leu Leu Asp Trp Val Lys 210 215 220	672	
TTC AAA GGC AAC CCG GTT CTG GTT CCT CCA CCC GGC ATT GGT GTC AAG Phe Lys Gly Asn Pro Val Leu Val Pro Pro Pro Gly Ile Gly Val Lys 225 230 235	720	
GAC TTT AGA GAC CCG ACT ACT GCT TGG ACC GGA CCA CAA AAT GGG CAA Asp Phe Arg Asp Pro Thr Thr Ala Trp Thr Gly Pro Gln Asn Gly Gln 240 245 250	768	
TGG CTG TTA ACA ATC GGG TCT AAG ATT GGT AAA ACG GGT GTT GCA CTT Trp Leu Leu Thr Ile Gly Ser Lys Ile Gly Lys Thr Gly Val Ala Leu 255 260 265 270	816	
GTT TAT GAA ACT TCC AAC TTC ACA AGC TTT AAG CTA TTG GAT GGA GTG Val Tyr Glu Thr Ser Asn Phe Thr Ser Phe Lys Leu Leu Asp Gly Val 275 280 285	864	
CTG CAT GCG GTT CCG GGT ACG GGT ATG TGG GAG TGT GTG GAC TTT TAC Leu His Ala Val Pro Gly Thr Gly Met Trp Glu Cys Val Asp Phe Tyr 290 295 300	912	
CCG GTA TCT ACT AAA AAA ACA AAC GGG TTG GAC ACA TCA TAT AAC GGG Pro Val Ser Thr Lys Lys Thr Asn Gly Leu Asp Thr Ser Tyr Asn Gly 305 310 315	960	
CCG GGT GTA AAG CAT GTG TTA AAA GCA AGT TTA GAT GAC AAT AAG CAA Pro Gly Val Lys His Val Leu Lys Ala Ser Leu Asp Asp Asn Lys Gln 320 325 330	1008	
GAT CAT TAT GCT ATT GGT ACG TAT GAC TTG GGA AAG AAC AAA TGG ACA Asp His Tyr Ala Ile Gly Thr Tyr Asp Leu Gly Lys Asn Lys Trp Thr 335 340 345 350	1056	



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CCC GAT AAC CCG GAA TTG GAT TGT GGA ATT GGG TTG AGA CTA GAC TAT Pro Asp Asn Pro Glu Leu Asp Cys Gly Ile Gly Leu Arg Leu Asp Tyr 355 360 365	1104
GGG AAA TAT TAT GCA TCA AAG ACT TTT TAT GAC CCG AAG AAA GAA CGA Gly Lys Tyr Tyr Ala Ser Lys Thr Phe Tyr Asp Pro Lys Lys Glu Arg 370 375 380	1152
AGA GTA CTG TGG GGA TGG ATT GGG GAA ACT GAC AGT GAA TCT GCT GAC Arg Val Leu Trp Gly Trp Ile Gly Glu Thr Asp Ser Glu Ser Ala Asp 385 390 395	1200
CTG CAG AAG GGA TGG GCA TCT GTA CAG AGT ATT CCA AGG ACA GTG CTT Leu Gln Lys Gly Trp Ala Ser Val Gln Ser Ile Pro Arg Thr Val Leu 400 405 410	1248
TAC GAC AAG AAG ACA GGG ACA CAT CTA CTT CAG TGG CCA GTG GAA GAA Tyr Asp Lys Lys Thr Gly Thr His Leu Leu Gln Trp Pro Val Glu Glu 415 420 425 430	1296
ATT GAA AGC TTA AGA GTG GGT GAT CCT ACT GTT AAG CAA GTC GAT CTT Ile Glu Ser Leu Arg Val Gly Asp Pro Thr Val Lys Gln Val Asp Leu 435 440 445	1344
CAA CCA GGC TCA ATT GAG CTA CTC CGT GTT GAC TCA GCT GCA GAG TTG Gln Pro Gly Ser Ile Glu Leu Leu Arg Val Asp Ser Ala Ala Glu Leu 450 455 460	1392
GAT ATA GAA GCC TCA TTT GAA GTG GAC AAA GTC GCG CTT CAG GGA ATA Asp Ile Glu Ala Ser Phe Glu Val Asp Lys Val Ala Leu Gln Gly Ile 465 470 475	1440
ATT GAA GCA GAT CAT GTA GGT TTC AGT TGC TCT ACT AGT GGA GGT GCT Ile Glu Ala Asp His Val Gly Phe Ser Cys Ser Thr Ser Gly Gly Ala 480 485 490	1488
GCT AGC AGA GGC ATT TTG GGA CCA TTT GGT GTC ATA GTA ATT GCT GAT Ala Ser Arg Gly Ile Leu Gly Pro Phe Gly Val Ile Val Ile Ala Asp 495 500 505 510	1536
CAA ACG CTA TCT GAG CTA ACG CCA GTT TAC TTT TAC ATT TCT AAA GGA Gln Thr Leu Ser Glu Leu Thr Pro Val Tyr Phe Tyr Ile Ser Lys Gly 515 520 525	1584
GCT GAT GGT CGT GCA GAG ACT CAC TTC TGT GCT GAT CAA ACT AGA TCC Ala Asp Gly Arg Ala Glu Thr His Phe Cys Ala Asp Gln Thr Arg Ser 530 535 540	1632
TCT GAG GCT CCG GGA GTT GGT AAA CAA GTT TAT GGT AGT TCA GTA CCT Ser Glu Ala Pro Gly Val Gly Lys Gln Val Tyr Gly Ser Ser Val Pro 545 550 555	1680
GTG TTG GAC GGT GAA AAA CAT TCA ATG AGA TTA TTG GTG GAT CAC TCA Val Leu Asp Gly Glu Lys His Ser Met Arg Leu Leu Val Asp His Ser 560 565 570	1728
ATT GTG GAG AGC TTT GCT CAA GGA GGA AGA ACA GTC ATA ACA TCG CGA Ile Val Glu Ser Phe Ala Gln Gly Gly Arg Thr Val Ile Thr Ser Arg 575 580 585 590	1776
ATT TAC CCA ACA AAG GCA GTA AAT GGA GCA GCA CGA CTC TTT GTT TTC Ile Tyr Pro Thr Lys Ala Val Asn Gly Ala Ala Arg Leu Phe Val Phe 595 600 605	1824
AAC AAT GCC ACA GGG GCT AGC GTT ACT GCC TCC GTC AAG ATT TGG TCA Asn Asn Ala Thr Gly Ala Ser Val Thr Ala Ser Val Lys Ile Trp Ser 610 615 620	1872
CTT GAG TCA GCT AAT ATT CAA TCC TTC CCT TTG CAA GAC TTG TAATCTCTT Leu Glu Ser Ala Asn Ile Gln Ser Phe Pro Leu Gln Asp Leu 625 630 635	1924
TATTTCTTTT TTTTTTCTT TTTCATTTGA AGGTTATTTT ACCGACGTCC CATCAAGAAA	1984
GGGAAGAGGG AGATCAATAT ATGTAGTGTT ATTCGCCCTA CCTTAGGATT AGATGTCATC	2044

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TAGCAATGTC AAATCTAGTA GAGTATACAA TGTATGGGTT CCTGGAAACC GAGTAGAGCT 2104  
TACCTGGATT CTATGTAAAC TAAGAAAGCT CAGCAATAT ATGCACAAAT AATTTACAGA 2164  
AACAACTTGG GAATGTTGAC AAAAAAAAAA AAAAA 2199

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10798 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Lycopersicon esculentum*

## (ix) FEATURE:

- (A) NAME/KEY: precursor\_RNA
- (B) LOCATION: 3520..7445

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCTCGATA AGTTATGTCT TGTGGAATC GATATCAAAT AACCGTCGAC GGTATCTTTG 60  
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CACTTAGAAA AGTGACATTT TGGACTGGTA GTCCATAAAT CAAGGTATAA TGTCAGTGGG 240  
GTACAAATAA ATTATTATGT GATAGTATAA CCGTAAGATA TCAAATACGG TTTGTGCCTT 300  
GGGGCATAAA GGTTTATCGC AAAAATCCTG ACATTATTGG AGATGTTTTT TCCTTTGGTG 360  
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AAATTGTAAT GAAGGTTATA TGAAAATCCT TGAACAATC CAGGTGTCTG AAGCATATAA 480  
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TTGGTAAGAC AAAATTTTGT CTTGACCTAC AGATTGTTAA TTTGACAAAT AAAATATTTG 660  
TCTAACAGAC AACAGTGCAC ATACACTGAA AAATTTTGAT GCAATTTTAT GTGGATATAT 720  
CGCATTCATT GAGTACCCCA ATGATTATGA GATCACTTGA CATAAATGAT GATTCAGTTT 780  
GATCTCAAAA GAAGGATAAG AGTTTCTTGG TGATGAACT CTATCTTGGT GCAATGAGGG 840  
CACTAGTGCA TCTTACTAAC AATATTTGAC TAGATATTTG TTTTGCACTA AATTTACTGG 900  
CAAGATTCAG TTTCTCCCGG ATAAAGGAC ATTGAAATGG TGTGAGCAC ATGAATGAAT 960  
ATCCTCAAAG GACCATAGTT ATGGGTTTAT TCTATCCCGA GGAATCCAAG ACAAATTTGA 1020  
TTGATTACGC AGATGCAGAA TATTTATCTG ATCCGCATAA AGCTCTATCT CAAGCACGCT 1080  
ATGTGTTTGC ATGTGGAGGC ACAATAATAT CCTGGGGATC AATGAAGCAA ATGTTGCTCT 1140  
GCAGAAATAA AAGTCTCCA TGAAGCAAGT CAAAAGTGGC TCTGGTTGAG ATAAATGACA 1200  
CACCATATTC AAGAAATGTG TGGTTTTTCT TAAAAAAG AATATACCAA CCACAATGTA 1260  
CAAAGATTGG AGACATCATC ACAAGAAATC AAGTGATGTT TTAATCAGGG GGAGTACAAT 1320  
ACGCGTTGCA CTCTTTTTC CTGATCGAG GTTTTTTTC CACTGGATTT TCCTGACAAG 1380  
GTTTTTAATG AGGCAACAAA TGGTGCGTAT CAAAAGATAT GTGTACTCTT TTTCTTCAC 1440  
TAGAATTTT TCCCACAGGG TTTTCTCTAG TAAGGTTTTA ACGAGGCACA TTATCTATGG 1500  
ACATCCAAGG GGGAGTGTTA TAAATACATT GAATTAAGTG GATAGTCCAT AAGGTTGGCA 1560

CATGAACAAC CATTCAATATT CACTAGGTGA CATGAACCTT TTTGGATAAG AATGTATCTA	1620
TTTATTATGA TACTTAATAT GGTAATCTTT GGAGTGATTI CTCACCTAT AAATAGAGTT	1680
GTTCAATCAC TATTGTAATA TATACATATG AGACTTGAAT ACACTTGAAT ACGAAGAAAAG	1740
TCTTATCTTC CATCTTACTT CTCTTGCTT CTCTCTTTAT GATTATATTC TTATGAGCTT	1800
GATTTTATAA CACGAATCTC ATTATACGAA AAGTTTTACT ATTTATATTT AATTAATAGA	1860
GGATTTAAAC TTTTAAATT TCTGTCTTTA TAGATGAGAA CTTGTCTTTT TGTGAATCC	1920
AACTAAACAT TCAATGAAGA CAAATCAACC TGTAAATCCC TTTCAAGTAG GATTTATTCG	1980
AATCTCATT TACGAAAAGT TTTACTATTT ATATTTAATT AATAGAGGAT TTAAACTTTT	2040
TAAATTTCTG TCTTTATAGA TGAGAACTTG TCTTTTGTG GAATCCAAC AAACATTCAA	2100
TGAAGACAAA TCAACCTGTA AATCCCTTTC AAGTAGGATT TATTCGAATC TCATTATACG	2160
AAAAGTTTAA CTATTTATAT TTAATTAATA GAGAATTTAA ACTTTTTAAA TTTCTGTCTT	2220
TATAGATGAG AACTTGCTCT TTTGTTGAAT CCAACTAAAC ATTCAATGAA TACAAATCAA	2280
CCTGTAAATC CCTTCAAGT AGGATTTATT CGAATCTCAT TATACGAAAA GTTTTACTAT	2340
TTATATTTAA TTAATAGAGA ATTTAAACTT TTTAAATTTT TGTCTTTATA GATGAGAACT	2400
TGTCTTTTTG TTGAATCCAA CTAAACATTC AATGAATACA AATCAACCTG TAAATCCCTT	2460
TCAAGTAGGA TTTATTCGAA TCTCATTATA CGAAAAGTTT TACTATTTAT ATTTAATTAA	2520
TAGAGAATTT AAACTTTTTA AATTTCTGTC TTTATAGATG AGAAGTTGTC TTTTGTGGA	2580
ATCCAACATA ACATTCAATG AATACAAATC AACCTGTAAA TCCCTTTCAA GTAGGATTTA	2640
TTCGAATCTC ATTATACGAA AAGTTTTACT ATTTATATTT AATTAATAGA GAATTTAAAC	2700
TTTTTAAATT TCTGTCTTTA TAGATGAGAA CTTGTCTTTT TGTGAATCC AACTAAACAT	2760
TCAATGAATA CAAATCAACC TGTAAATCCC TTTCAAGTAG GATTTATTCG AATCTCATT	2820
TACGAAAAGT TTTACTAGTT ATATTTAATT AATATTCAAG TCTCAATTTT TTTTAAATA	2880
TTTACATTCC ACATTTTAAT CTATAATGAA AGTTACTAAA ATATACTATC AAGGAGAAAA	2940
TATACAAAAT GGCCCATAC GATAGTCTTT AATATATAAT AAATATGTTT ATTTGGATCC	3000
TTAATATATT TCACCTGATT AAAATAATAA TAAATGTATA ATAAAAAGTG GTCATTTTGG	3060
TCTTTTGTCC TAAACATAGA GTTTTTTTAC CTCAAAGAA AAATCTTCCA TAAATCTAA	3120
TACTATTTTT TTTAATTTT TCCAACAAAA TTTATTATTT TCTCTTTTAA ATATTATTTT	3180
ACTGACCTAA TAACAGTTTT TATTTTGAGC AAGAAAAGTA GTAAATTTTG TTAATAAAG	3240
AACCAAAATA AATCATTTTA ATCAAAGTAA AATATAATAA CGATTAAAT AAAGTATACA	3300
TTAAGTCATT TCAATGAAGT GAAATAAATG AAGAAGTAAA ATAAAAAAT TAACCAACA	3360
GTAAGCATAG TTTTGGTCAT TTTCTCTAAT CCAAGTGTA CCTCAAATTA TAAAGTCCT	3420
TTTGTACTC AATTTCTGTT GTCCAGTCA TTTTCTGTGT TCATCACCTA TATATATAGC	3480
AGTAGACTAG TAGCTTCTCC CATTCTCTA TCTTCTATTA TGGCCACTCA GTGTTATGAC	3540
CCCGAAAAC CCGCTCTCG TTACACATTA CTCCCGATC AACCCGATC CGGCCACCGG	3600
AAGTCCCTTA AAATCATCTC CGGCATTTTC CTCTCCGTTT TCCTTTTGCT TTCTGTAGCC	3660
TTCTTTCCGA TCCTCAACAA CCAGTCACCG GACTTGCAA TCGACTCCCG TTCGCCGGCG	3720
CCGCCGTCAG GAGGTGTTTC TCAGGGAGTC TCCGATAAAA CTTTTCGAGA TGAGCCGGT	3780
GCTAGTCACG TTTCTTATGC GTGGTCCAAT GCTATGCTTA GGTGGCAAAG AACGGCTTAC	3840
CATTTTCAAC CTCAAAAAA TTGGATGAAC GGTAAATTAAC TTTCTTATTT TGACTTTTCT	3900

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GAATGAGCAA ATCATTAAAT CGGGTGCTTA TGTATATCAT CTCGGTTAAT CCTTTACCT 4200  
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GCTGATACAT GAGTCTGATG TGGCAGATAC ATTAATCTGA TAGGTAAAAA TGAGGAACTA 5100  
GAAATTTATA AAATAATAT GAATAATGAT AATAAGATAA CTAAATGTG AAATTTCTAT 5160  
CATTCTCCT AACATAACCAC TAGTGAAATT TGTTTACGTA TCTTGTGAA GAAAATCTTA 5220  
TCCAAAAGTC AAAAATAAAA ACTCGTGGCC AAATTTTCAA AAAAAAAGA AGGTTATCTT 5280  
TTTGCCGCAA AAAGCATAGC AATTTTGGA CGGAACGTAT TGAGATTTTG TAGAGTATTT 5340  
TATAATTCOA ATTGCATAGA AAAGTCTTAC CTATACAAGT AAAAATTTG AAATTTCTAT 5400  
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ATACCGGTGA CACTGATGAT TATGTGCAAG TGCAAAATCT TGCGTACCCC GCCAATTTAT 5700  
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CCGGCATTGG TGTCAAGGAC TTTAGAGACC CGACTACTGC TTGGACCGGA CCACAAAATG 5820  
GGCAATGGCT GTTAACAATC GGGTCTAAGA TTGGTAAAC GGGTGTGCA CTGTTTATG 5880  
AAACTTCCAA CTTCACAAGC TTTAAGCTAT TGGATGGAGT GCTGCATGCG GTTCCGGGTA 5940  
CGGGTATGTG GGAGTGTGTG GACTTTTACC CGGTATCTAC TAAAAAACA AACGGGTGG 6000  
ACACATCATA TAACGGGCCG GGTGTAAAGC ATGTGTAAAG AGCAAGTTA GATGACAATA 6060  
AGCAAGATCA TTATGCTATT GGTACGTATG ACTTGGGAAA GAACAAATGG ACACCCGATA 6120  
ACCCGGAATT GGATTGTGGA ATTGGGTGGA GACTAGACTA TGGGAAATAT TATGCATCAA 6180  
AGACTTTTTA TGACCCGAAG AAAGAACGAA GAGTACTGTG GGGATGGATT GGGGAAACTG 6240

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CATGGTAGGG AAATTTCTTA TTTAGAGAAA GTTGTATATA TCAATGCATT TGAGGTGAA	6420
GTAATTCTG AATTGTATAT GAAACGTGTC TAATAGTGT TCGAAATAAC AGAGTATTC	6480
AAGGACAGTG CTTTACGACA AGAAGACAGG GACACATCTA CTTCAGTGGC CAGTGAAGA	6540
AATTGAAAGC TTAAGAGTGG GTGATCCTAC TGTAAAGCAA GTCGATCTTC AACCAGGCTC	6600
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TAGGTTTCAG TTGCTCTACT AGTGGAGGTG CTGCTAGCAG AGGCATTTTG GGACCATTG	6840
GTGTCATAGT AATTGCTGAT CAAACGCTAT CTGAGCTAAC GCCAGTTTAC TTTTACATT	6900
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TTCTATCTGG CACAATTAAT TTGTCCTTGT AAAATGGAGA TGGATAAAAG TAGCGGGTTG	7020
TTGATCTGAT ATATGCAGAT CCTCTGAGGC TCCGGGAGTT GGTAACAAG TTTATGGTAG	7080
TTCACTACCT GTGTTGGACG GTGAAAAACA TTCAATGAGA TTATTGGTAA GTGATAATGA	7140
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CCTCCGTCAG GATTTGGTCA CTTGAGTCAG CTAATATTCA ATCCTTCCCT TTGCAAGACT	7440
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GAGCAACTTG GTAAAAAGTA TATGGTATAA GTAAGATATT TTTAAATGTA TTATGTATCA	7980
GTTGTACTCA ATCAAAGAGC GGATAAATAC AATTGATACA ATATACAAAA TAGTTATGCA	8040
CTAAATAATA AATAGAGGAT AAAATGTAAA AGAAATACAA AATATAATTC TCTCGATCTC	8100
GCTCCCGTCT CTCCTCTCTC GATCTCACTC ATCTCTCTTC TCTTAATATG TATTCATTTT	8160
AATACAAAT AGTTTCTATT TGTATTTTTT CTTCAAAAT CACGAAAAA AATATATATA	8220
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ATGGAATACT TTTAAGGGTT AATGTTTGT GTTTTTGAAA TTTCCCTCT TGAAGCCCTT	8340
AAGTGCAAT CTTGAATCCA CTATGAATAT GATTCATTCT TTATACATAT ACAATAATAA	8400
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GAAGATCAGC CCAGCCCATC TAATTCAAGC CTCGTGGGCC AAGAAATTTA ATGAGCTAAG	8520
GAAGTTGGC CCTTTATTTG AAAGTGCTA AATTGTTCAA CTCAACCTAA TTTTAGAAGG	8580

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 ACAAAAAATT TGTATGATTA ACATGTACCT TGGATACTAC ATGCCCAAGC TACATGTATA 8760  
 TGTGTGATG CATTCCAAAT ATGCAAGCGA GATAAGAGCG ACCAAGATGG GTGGGAGGCG 8820  
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 AAATATGAC TATAACATTC AAATATAATT TTTATGTTTG CCATATATGA AAATTGATCT 10260  
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 GAATTGACAG AACCATTGTA CCATTCTTCT CATAGTAAA ATAGTATATA ATTGGGCTCG 10680  
 ACTTTATATA AAATTCTGAT ATATTATTTA ATATTCTTCT TTGCTTTTCC TTTTCTGCAT 10740  
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4032 base pairs  
 (B) TYPE: nucleic acid

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(C) STRANDEDNESS: double  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Lycopersicon esculentum*

(ix) FEATURE:  
(A) NAME/KEY: precursor\_RNA  
(B) LOCATION: 889

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCAAAATT AGTTTGCAC TCTTCTCAC ATTTCAAGCA TTAACACCTGT	60
TTTATTATTA TTATTATATT AATTTAAATT TTCTGAGTTT AATTTTATTA TTCTAACATT	120
ATTTTATATA CTTTTCATTG AAAAATTGCA TTGTTTATAT TCTTACTTCA TAATGTACGT	180
ATATAACATT CTTTGCAGAC TTCATTTATG AAATTACACT ATAGAATAAT AATTTGATT	240
ATATGTACTT CCTTCCTTTC AAATTGATTA AATTGTTAAG GTGTTTCACA CATTTAAAT	300
AAATTAAGTC ACATATTAAG CATAACTTTA AATTTTACA AAAATAAGAG CTCTCTATAA	360
AGTTTGACTT TAAGTCTCCA AATTTGTAA TACAGACCTG AAAGAGTGTA GGAGCTAACA	420
AAACAAATAG TTATAAAAAG TAATTTTATT CAATTTTATA GAATTAAGA CTATATGTGC	480
ATACACCAAA ATTTTACATC CTTTATCATA GCAAAATTTA TAGAAAATAA AAATAAATTT	540
GTAACATAATG TTTTTTTTTT CAAACACTGT AAAACACGAA AAAAATTGCT AATGTGTAAG	600
AAAACATGTG TAATATAAAA CAAATATAAA AGAGTCCACG TGCATCGCAT GAGTACCTAT	660
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TTCAACCAAA AAAAAAAGA AGGTTAAAC GCAAAGCAAC TTAATTCATT TGTATAAAT	840
TGGAGGAGCC AAAGATAGTG AGATTCACAA AACTTTATAT CTCTAAGAAT GGAAATTCAA	900
AAGGTATCAT AGTTTCTAAT ATTTTTTTTA ATTATATATG TCTATCTTAA GTTTCATTCA	960
TATACTCATG ATTAATTTAT TGATCATTTT AAACAATGAA ACATATCTTA GATTTAATTT	1020
TATTTATTTA TTTTATAAAC ATAGGAGTTT GATTTAACGA TAGTTCCAAC AGAAGGTGAA	1080
ATTGATGCAC CATCATCGCC AAGGAAGAAT TTATGTCTCA GTGTGATGGA ATCTGATATT	1140
AAAAATGAAA CGTCTTTTCA AGAACTCGAC ATGATTTTGA CTCAATATTT AGAGACATTG	1200
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TCTCAATTTT ATTTAAATAA ATACATTTTC ATTTATTTT ACGTGTGTAA TAAACATAAA	1380
AGTATTTATA AGAAAAATTA ATCAAAAGTT ATTCATTAAT AAATCATCCC TAACTTTATT	1440
TTTACATATC TTTTAAGTAT TTTTGATTG GCCAAATAAT ATTTTACGAT TTTATTATA	1500
ATTATATCTT TGTTATTTA ATTTACAGGT TATCCAATTA ACATGTGTTA CGAACATCAT	1560
GCCACTTTAG CCCCACTTTT GCAATTTTAT TTGAACAATT GTGGAGATCC CTTTACTCAG	1620
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CTCTGGGAAA TAGAGAAAGA TGAATATTGG GGNTACATTA CTAGTGGTGG CACTNAGGGC	1740
AATCTCCATG GCCTTTTGGT TGGGCAGGTA TCATTTTCAA GAAAGGGGGT GGGGGGAGAG	1800
GTGGTAGTTT TTGAATCATA TGAAAAATCA AAAAATTAAT TGGCGTAATC AGCCATTGTC	1860
ATGGTCAAAA TCATTACGAG CAAGACGTCT TACTTTACTT TTGTTGTACC ATAGGTACAC	1920

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TCAACAGATG GATTTCAAAG TTATCACATG TTGCTTCCAT ATTCTTTTCA AGGAATGGAG 2700
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AACTCGAATT GATGCTTAC CCTCTTGGTG CGATAGAAT TGAATCTACC GTCTTACCCT 2940
CAAATATTTT TCATAATGAA TGACATAAAT GAGTCTTTTT TAAACAAATT TGATAACATA 3000
TTTGAGTTTT TTTCTTATGG TTAAATGATG CAAGTGCTTC ATCACTTTCA TAAAGCATT 3060
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CAAATGACAA TTATTATATC CATTGCGATG CAGCATTATG TGGGCTAATT CTCCCATTTA 3480
TCAAACATGT AAGCTTATTT TTATTCAATT TTCCTTCAAC GCTCGATCGA AGTTACAATG 3540
ACATAGTTTC TTTCTATGGT ATTTGACAAT AGGCAAAAAA AATTACCTTC AAGAAACCAA 3600
TTGGAAGTAT TTCAATTTCA GGGCACAAAT TCTTGGGATG TCCAATGTCT TGTGGCGTTC 3660
AGATAACAAG GAGAAGTTAC GTTAGCACCC TCTCAAAAT TGAGTATATT AATTCCGCAG 3720
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GCAAGAAAGG ACATGCTAGA TTGCAACAAG ATTCATAAC ATGCATTGAA AATGCTCGGT 3840
ATTTGAAAGA TCGACTTCTT GAAGCAGGAA TTAGTGTAT GCTGAATGAT TTTAGTATTA 3900
CTGTTGTTTT TGAACGACCT TGTGACCATA AATTCATTCG TCGTTGGAAC TTGTGTTGCT 3960
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TCTTCAAAGA TC 4032

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## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10965 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: unknown



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(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Lycopersicon pimpinellifolium*

(ix) FEATURE:

(A) NAME/KEY: precursor RNA

(B) LOCATION: 3686..7612

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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ATAGTGTGAA CAGATAAATG GTTGGCCAAG TAAAATGCAC AATTCAAGTA TATTTTGT	180
CACCTAGAAA AGTGACATTT TGGACTGGTA GTCCATAAAT CAAGGTATAA TGTCAGTGGG	240
GTACAAATAA ATTATTATGT GATAGTATAA CCGTAAGATA TCAAATACGG TTTGTGCCTT	300
GGGGCATAAA AGTTTATCGC AAAAATCCTG ACATTATTGG AGATGTTTTT TCCTTTGGTG	360
GATGCAATGA GGTITGTTTT GATCTGGCAA CATATGAAAA ACTTGAATGC ATGTAATGAA	420
AAATTGTAAT GAAGGTATA TGAAAATCCT TGAAACAATC CAGGTGCTG AAGCATATAA	480
AGGTGAAAG AAACCTATCC AATAAAGCTT CAAGAATCCT TATATGGATT GAAATAGTCA	540
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TTGGTAAGAC AAAATTTTGT CTTGACCTAC AGATTGTAA TTTGACAAAT AAAATATTTG	660
TCTAACAGAC AACAGTGAC ATACACTGAA AAATTTTGAT GCAATTTTAT GTGGATATAT	720
CGCATTCAAT GAGTACCCCA ATGATTATGA GATCACTTGA CATAAATGAT GATTCAGTTT	780
GATCTCAAAA GAAGGATAAG AGTTTCTTGG TGATGAACT CTATCTTGGT GCAATGAGGG	840
CACTAGTGCA TCTTACTAAC AATATTTGAC TAGATATTTG TTTTGAGTA AATTACTGG	900
CAAGATTCAG TTTCTCCCG ATAAAAGGAC ATTGAAATGG TGTGAGCAC ATGAATGAAT	960
ATCCTCAAAG GACCATAGTT ATGGGTTTAT TCTATCCCGA GGAATCCAAG ACAAATTTGA	1020
TTGATTACGC AGATGCAGAA TATTATCTG ATCCGCATAA AGCTCTATCT CAAGCACGCT	1080
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GCAGAAATAA AGTCTCTCA TGAAGCAAGT CAAAAGTGCG TCTGGTTGAG ATAAATGACA	1200
CACCATATTC AAGAAATGTG TGGTTTTTCT TTAATAAAG AATATACCAA CCACAATGTA	1260
CAAAGATTGG AGACATCATC ACAAGAAATC AAGTGATGTT TTAATCAGGG GGAGTACAAT	1320
ACGCGTTGCA CTCTTTTCC CTTGATCGAG GTTTTTTCC CACTGGATT TCTGACAAG	1380
GTTTTTAATG AGGCAACAAA TGGTGGTAT CAAAAGATAT GTGTACTCTT TTTCTTCAC	1440
TAGAATTTT TCCACAGGG TTTTCTAG TAAGGTTTGA ACGAGGCACA TTATCTATGG	1500
ACATCCAAGG GGGGGTGTTA TAAATACATT GAATTAAGTG GATAGTCCAT AAGGTTGGCA	1560
CATGAACAAC CATTCATATT CACTAGGTGA CATGAACCTT TTTGGATAAG AATGTATCTA	1620
TTTATTATGA TACTTAATAT GGAATCTTT GGAGTGATTT CTCACTCTAT AAATAGAGTT	1680
GTTCATTAC TATTGTAATA TATACATATG AGACTTGAAT ACACTTGAAT ACGAAGAAAG	1740
TCTTATCTT CATCTTACTT CTCTTGCTT CTCTCTTAT GATTATATC TTATGAGCTT	1800
GATTTTATAA CACGAATCTC ATTATACGAA AAGTTTACT ATTTATATTT AATTAATAGA	1860
GGATTTAAAC TTTTAAATT TCTGTCTTA TAGATGAGAA CTTGTCTTTT TGTGGAATCC	1920
AACTAAACAT TCAATGAAGA CAAATCAACC TGTAATCCC TTTCAAGTAG GATTTATTCG	1980

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AATCTCATT	TACGAAAAGT	TTTACTATTT	ATATTTAATT	AATAGAGSAT	TTAAACTTTT	2040
TAAATTTCTG	TCTTTATAGA	TGAGAACTTG	TCTTTTTGTT	GAATCCAAC	AAACATTCAA	2100
TGAAGACAAA	TCAACCTGTA	AATCCCTTC	AAGTAGGATT	TATTCGAATC	TCATTATACG	2160
AAAAGTTT	CTATTTATAT	TTAATTAATA	GAGAATTTAA	ACTTTTTAAA	TTTCTGTCTT	2220
TATAGATGAG	AACCTGTCTT	TTTGTGGAAT	CCAACATAAC	ATTCAATGAA	TACAAATCAA	2280
CCTGTAAATC	CCTTCAAGT	AGGATTTATT	CGAATCTCAT	TATACGAAAA	GTTTTACTAT	2340
TTATATTTAA	TTAATAGAGA	ATTTAACTT	TTTAAATTC	TGTCTTTATA	GATGAGAACT	2400
TGTCTTTTTG	TTGAATCCAA	CTAAACATTC	AATGAATACA	AATCAACCTG	TAAATCCCTT	2460
TCAAGTAGGA	TTTATTCGAA	TCTCATTATA	CGAAAAGTTT	TACTATTTAT	ATTTAATTAA	2520
TAGAGAATTT	AACTTTTTTA	AATTTCTGTC	TTTATAGATG	AGAACTTGTC	TTTTTGTGGA	2580
ATCCAACATA	ACATTCATG	AAGACAAATC	AACCTGTAAA	TCCCTTTCAA	GTAGGATTTA	2640
TTCGAATCTC	ATTATACGAA	AAGTTTTACT	ATTTATATTT	AATTAATAGA	GAATTTAAAC	2700
TTTTTAAAT	TCTGTCTTTA	TAGATGAGAA	CTTGCTTTTT	TGTTGAATCC	AACTAAACAT	2760
TCAATGAATA	CAAAATCAACC	TGTAAATCCC	TTTCAAGTAG	GATTTATTCG	AATCTCATT	2820
TACGAAAAGT	TTTACTATTT	ATATTTAATT	AATAGAGAAT	TTAAACTTTT	TAAATTTCTG	2880
TCTTTATAGA	TGAGAACTTG	TCTTTTTGTT	GAATCCAAC	AAACATTCAA	TGAATACAAA	2940
TCAACCTGTA	AATCCCTTC	AAGTAGGATT	TATTCGAATC	TCATTATACG	AAAAGTTTTA	3000
CTATTTATAT	TTAATTAATA	TTCAAGTCTC	AATTTTTTTT	TAAATATTTA	CATTCCACAT	3060
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CATAACGATA	GTCTTTAATA	TATAATAAAT	ATGTTCAATT	GGATCCTTAA	TATATTTAC	3180
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CATAGAGTTT	TTTTACCTTC	AAAGAAAAAT	CTTCATAAA	ATCTAATACT	ATTTTTTTTT	3300
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AGTTTTTATT	TTGAGCAAGA	AAAGTAGTAA	ATTTTGTTAA	ATAAAGAACC	AAAATAAATC	3420
ATTTAATCA	AAGTAAATA	TAATAACGAT	TAAATAAAG	TATACATTAA	GTCATTTCAA	3480
TGAAGTGA	TAAATGAAGA	AGTAAATAA	AAAAATTAAC	CAACAGTAA	GCATAGTTT	3540
GGTCATTTTC	TCTAATCCCA	AGGTACCTC	AAATTATAAA	AGTCCTTTTG	TTACTCAATT	3600
TCGTTGGTCC	CAGTCATTTT	CTGTGTTTCT	CACCTATATA	TATAGCAGTA	GACTAGTAGC	3660
TTCTCCCAT	CTTCTATCTT	CTATTATGGC	CACTCAGTGT	TATGACCCCG	AAAACCTCCG	3720
CTCTCGTTAC	ACATTACTCC	CGGATCAACC	CGATCCGGC	CACCGGAAGT	CCCTTAAAT	3780
CATCTCCGGC	ATTTTCTCT	CCGTTTTCT	TTTGCTTTCT	GTAGCCTTCT	TTCCGATCCT	3840
CAACAACCG	TCACCGGACT	TGCAATCGA	CTCCGTTTCG	CCGGCGCCGC	CGTCAAGAGG	3900
TGTTTCTCAG	GGAGTCTCCG	ATAAACTTT	TCGAGATGTA	GCCGGTGCTA	GTCACGTTTC	3960
TTATGCGTGG	TCCAATGCTA	TGCTTAGCTG	GCAAGAACG	GCTTACCATT	TTCAACCTCA	4020
AAAAATYGG	ATGAACGGTA	ATTAACCTTC	TTATTTTGAC	TTTTCTTTAA	TTTCTTTTTT	4080
ATTTGATCTT	AAAATTGAAA	TTATTTATAA	ATACTTATAA	CAGTTCTTTT	TTTTCTCAAT	4140
GATATTTATG	GCTATTGATC	TGTTGGGGGT	ATCTTTTGGA	TTCTGATTGG	ATGCTATTCT	4200
GCAGATCCTA	ATGGTGAGTT	CAAAGTTAAT	TATTATCACT	ATTTTCTGCT	AGTTTTTAAT	4260
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TTAATTCGGG TGCTTATGTA TATCATCTCG GTTAATCCTT TTACCTTATA CTCAAAAACA 4380  
AATATTACTC CCTTCAAAAT AATTGATGTT TGACATAATC AATGTGATGT TTAATTTTTT 4440  
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CACAAGCTTT AAGCTATTGG ATGGAGTGCT GCATGCGGTT CCGGGTACGG GTATGTGGGA 6120  
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TGAAGTGGAC AAAGTCGGCG TTCAGGGAAT AATTGAAGCA GATCATGTAG GTTTCAGTTG 6960  
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TTTTCAATC AAATATGGTA ACAATGGTGT TTTTCAATA AACTAACA AAAATTTGTA 8880  
TGATTAACAT GTACCTTGA TACTACATGC CCAAGCTACA TGTATATGTT GTGATGCATT 8940  
CCAAATATGC AAGCGAGATA AGAGCGACCA AGATGGGTGG GAGGCGAGGG CTTGGAATTT 9000

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GTTTATATAT CCTAGATACA TGCGAATCCA TTTGAATGAA GTCCTTCTAG AATAAATAGA 9060  
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 GTTGAATGGT CTTGTAAATC CTATCACAAC CATACCCCAA TACCGAGTTG GGTGGACCG 9300  
 GCTCCATGGG CTTAGCAAAC TTTGACATAT CTACACATAA TGGAACAAAT GAAAAAAAAA 9360  
 ATACGAAATG AAATTATTTT TAAACAATA AAGACAATAT TTTTITAGAG AAAGTTACAA 9420  
 AATTATATAC AACTTAATAT TATTATATCC TCTAAAAATT CCTATCTTTG AATTAAATAC 9480  
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 AACCCCAATT ATTTCAAATA TAATTATATT AGTGATACAC AACTTGTTTA TTGTTTGTTA 9660  
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 TTCTGATATA TTATTTAATA TTCTTCTTG CTTTCTCTT TCTGCATTAC TTTTCTTTC 10920  
 CATTTAATA ATAATACAGG TTTATGGGTA TTATAAACG GATCC 10965

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATTCTAGAA GATAGAGGAA TG

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## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGAAGCTTAA TCAACCTGTA AATCCC

26

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCATGCTC CGTCCTGTAG

20

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTGCATGCCT GCAGTTGTTT GCCTCCCTGC TG

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## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AACTGCAGAA TGGAGCAGCA CGACTC

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THAT WHICH IS CLAIMED IS:

1. Isolated substantially pure DNA, comprising a sequence of nucleotides that encodes a tomato vacuolar invertase.

5        2. Isolated substantially pure DNA, comprising a sequence of nucleotides that hybridizes to the DNA of claim 1 and that encodes invertase.

3. The DNA of claim 1, wherein said tomato vacuolar invertase is *Lycopersicon esculentum* or *Lycopersicon*  
10 *pimpinellifolium* invertase.

4. The DNA of claim 3, wherein the DNA that encodes invertase is genomic DNA.

5. The DNA of claim 3, wherein the DNA that encodes invertase is cDNA.

15        6. A substantially pure DNA fragment encoding a tomato fruit invertase, comprising the amino acid sequence set forth as residues 1-636 in Sequence ID No. 1.

7. The DNA fragment of claim 6, wherein the DNA  
20 encoding the tomato fruit invertase has substantially the same nucleotide sequence as set forth in Sequence ID No. 1.

8. The DNA fragment of claim 5, wherein the DNA encoding invertase has substantially the same nucleotide  
25 sequence as the portion of nucleotides 1-2199 set forth in Sequence ID No. 1 that encodes invertase.

9. Isolated DNA that hybridizes to the DNA of Sequence ID. No. 1 and that encodes invertase.

10. Isolated DNA that hybridizes to the DNA of  
30 Sequence ID. No. 2 and that encodes invertase.

11. Isolated DNA that hybridizes to the DNA of Sequence ID. No. 4 and that encodes invertase.

12. The DNA of claim 3 that does not include nucleotides of the sequence:

35

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5'-AAACAACCTTG GGAATGTTGA C-3',

which occur at residues 2164-2184 in Sequence ID No. 1.

13. A DNA construct, comprising the DNA of claim 1  
that encodes invertase operatively linked to a promoter  
5 which effects expression of heterologous genes in plants.

14. A DNA construct, comprising the DNA of claim 1  
that encodes invertase operatively linked to a  
developmentally regulated promoter, wherein said DNA  
encoding invertase includes sequences that encode a  
10 vacuolar targeting sequence.

15. The DNA construct of claim 14, wherein said  
promoter region is the HDC promoter region.

16. The DNA construct of claim 14, wherein the  
promoter region includes a sufficient portion of the  
15 sequence of nucleotides set forth in residues about 1-889  
of sequence ID No. 3 to effect developmentally regulated  
expression of the DNA encoding invertase.

17. The DNA construct of claim 13, wherein the  
promoter region is the promoter region from the  
20 *Lycopersicon pimpinellifolium* or *Lycopersicon esculentum*  
gene that encodes tomato fruit vacuolar invertase.

18. The DNA construct of claim 13, wherein said  
promoter is constitutively expressed and the DNA that  
encodes invertase includes sequences of nucleotides that  
25 a encode a vacuolar targeting sequence.

19. The DNA construct of claim 18, wherein the  
promoter is the cauliflower mosaic virus 35S promoter.

20. A DNA construct, comprising DNA encoding all or  
a portion of antisense invertase RNA operatively linked  
30 to a promoter, wherein said portion is sufficient to  
produce mRNA which is effective for inhibiting  
translation of mRNA that encodes the invertase and said  
promoter is developmentally regulated or is a  
constitutive promoter and effects transcription of  
35 heterologous genes in plants.



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21. A DNA construct, comprising DNA encoding a portion of invertase mRNA operatively linked to a promoter, wherein said portion encodes a truncated protein that is effective, upon introduction into a host  
5 cell, to cosuppress expression of endogenous invertase.

22. A method for increasing the soluble solids content of tomato fruit, comprising introducing the DNA construct of claim 13 into a plant cell to produce a transgenic plant that produces said fruit, whereby the  
10 soluble solids content of the fruit, at harvest, is greater than that in tomato fruit produced by a wild type plant of the same species.

23. The method of claim 22, wherein said invertase is a tomato fruit invertase.

15 24. The method of claim 22, wherein the onset of expression of said invertase commences at an earlier stage of ripening of the tomato fruit than occurs when said plant does not express said invertase, and said expression continues throughout the ripening of the  
20 tomato fruit.

25. A method for altering the solids content of tomato fruit produced by a tomato plant, comprising introducing DNA encoding antisense tomato fruit invertase mRNA or DNA encoding a truncated form of said invertase  
25 into tomato plant cells to produce a transgenic tomato plant, whereby the tomato plant expresses sufficient levels of antisense tomato fruit invertase mRNA or truncated invertase to reduce the total amount of tomato fruit invertase expressed in said plant such that the  
30 total amount of tomato fruit invertase in said transgenic plant during fruit ripening, is less than the amount of tomato fruit invertase produced by a tomato plant, which does not express antisense invertase mRNA or truncated invertase and which is a member of the same species as  
35 the transgenic plant.

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26. A transgenic plant, comprising the DNA of claim 1.

27. The transgenic plant of claim 26, wherein said plant is a tomato plant.

5 28. A plant protoplast, comprising the DNA of claim 1.

29. The plant protoplast of claim 28, wherein said protoplast is a tomato plant protoplast.

10 30. A tomato fruit produced by a transgenic tomato plant of claim 27.

31. The tomato fruit of claim 30, wherein said transgenic tomato plant is a species of *Lycopersicon esculentum* or *Lycopersicon pimpinellifolium*.

15 32. A seed produced by the transgenic plant of claim 27, wherein said seed contains DNA that encodes heterologous invertase.

33. A method for the recombinant production of tomato fruit invertase, comprising expressing, in a recombinant host, DNA encoding a tomato fruit invertase  
20 having substantially the same amino acid sequence as the amino acid sequence set forth in Sequence ID No. 1.

34. An invertase promoter region encoded by the DNA of claim 4.

25 35. The promoter region of claim 34, included in nucleotides 1-3519 of Sequence ID No. 2.

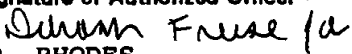
36. The promoter region of claim 34, included in nucleotides 2772-3519 of Sequence ID No. 2.

37. The promoter region of claim 34, included in nucleotides 2440-3519 of Sequence ID No. 2.

30 38. The promoter region of claim 34, included in nucleotides 1-3679 of Sequence ID No. 4.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01385

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C12P 21/00; C12N 5/10, 15/09; A01H 5/00; C07H 15/12 US CL : 435/69.1, 172.3, 240.4; 536/27; 800/205		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/69.1, 172.3, 240.4; 536/27; 800/205; 935/35, 60, 64	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, DIALOG search terms: invertase or fructosidase, tomato or lycopersicon, vacuol?, cosuppress?, histidine decarboxylase		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X/Y	The Plant Cell, Volume 2, issued November 1990, Sturm et al, "cDNA cloning of carrot extracellular B-fructosidase and its expression in response to wounding and bacterial infection", pages 1107-1119, see page 1109.	2,9-11/20
Y	US, A, 4,801,540 (Hiatt et al) 31 January 1989, see entire document.	1-14,16-20,22-25,33
Y	The EMBO Journal, Volume 9, No. 10, issued October 1990, Schaewen et al, "Expression of a yeast-derived invertase in the cell wall of tobacco and Arabidopsis plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants", pages 3033-3044, see entire document.	18-19
Y	Plant Cell Physiology, Volume 31, No. 5, issued May 1990, Endo et al, "Size and levels of mRNA for acid invertase in ripe tomato fruit", pages 655-659, see entire document.	1-14,16-19,22-38
Y	US, A, 4,394,443 (Weissman et al) 19 July 1983, see entire document.	1-14,16-19,22-38
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
13 MAY 1992		01 JUN 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		 P. RHODES

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,446,235 (Seeburg) 01 May 1984, see entire document.	1-14, 16-19, 22-38
Y	US, A, 4,943,674 (Houck et al) 24 July 1990, see entire document.	1-14, 16-19, 22-38
Y	Phytochemistry, Volume 14, issued 1975, Manning et al, "Distribution of acid invertase in the tomato plant", pages 1965-1969, see entire document.	22-25

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_, because they relate to subject matter (1) not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
  
3. ☐ Claim numbers \_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	Biochimie, Volume 70, issued 1988, Lauriere et al, "Characterization of B-fructosidase, an extracellular glycoprotein of carrot cells", pages 1483-1491, see page 1484, first full paragraph and page 1490, second and fourth full paragraphs.	1-14, 16-19, 26-32
A	Trends in Biotechnology, Volume 8, issued December 1990, Jorgensen, "Altered gene expression in plants due to <u>trans</u> interactions between homologous genes", pages 340-344, see entire document.	21

## FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

## VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-13, 22-24, 26-32, drawn to a first product and process of use, classified in Class 536 and 800, subclass 27 and 805, respectively, for example.

species (a) claims 14-16

(b) claim 17

(c) claims 18-19

II. Claim 20, drawn to a second product, classified in Class 536, subclass 27, for example.

III. Claim 21, drawn to a third product, classified in Class 536, subclass 27, for example.

IV. Claim 25, drawn to a method of using the second product, classified in Class 435, subclass 172.3, for example.

V. Claim 33, drawn to a second method of using the first product, classified in Class 435, subclass 69.1, for example.

VI. Claims 34-38, drawn to a fourth product, classified in Class 536, subclass 27, for example.

I. Claims 1-13, 22-24, 26-32, drawn to first product consisting of a coding region and constructs and plants containing same and a first method of using same, classified in Classes 435, 536, and 800, subclasses 172.3 and 240.4, 27, and 205, respectively, for example.

The following are independent and distinct species pertinent to the invention of Group I where a) is the first species and will be searched with claims 1-13, 22-24, and 26-32 in the event that no other fees are paid. Note that a search of any other additional species within Group I requires payment of additional fees.

a). HDC promoter (claims 14-16)

b). invertase promoter (claim 17)

c). constitutive promoter (claims 18-19).

II. Claim 20, drawn to second product consisting of antisense DNA, classified in Class 536, subclass 27, for example.

III. Claim 21, drawn to third product consisting of coding region for truncated protein, classified in Class 536, subclass 27, for example.

IV. Claim 25, drawn to method of using second product, classified in Class 435, subclass 172.3, for example.

V. Claim 33, drawn to second method of using first product, classified in Class 435, subclass 69.1, for example.

VI. Claims 34-38, drawn to fourth product consisting of a promoter, classified in Class 536, subclass 27, for example.

The inventions are distinct, each from the other because of the following reasons:

The products of Groups I-III and VI are unrelated and mutually exclusive inventions and are capable of separate manufacture and use and have different properties as claimed and thus are distinct inventions. The methods of Groups I and IV-V are also unrelated and mutually exclusive inventions involving different method steps and results as well as different products of use and products made and are distinct. The species a)-c) of Group I are unrelated and mutually exclusive and capable of separate manufacture and use and have different properties as claimed and confer different properties on the DNA constructs incorporating the species and are distinct.

Inventions of Group II and Group IV are related as product and process of use; the inventions of Group I and Group V are also related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. In the instant case each of the products of Groups I and II can be used in a materially different process such as molecular probes in hybridization reactions or as primers for PCR and DNA sequencing reactions, for example.

These inventions are distinct for the reasons given above and have acquired a

**FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS**

separate status in the art as shown by their different classification, recognized divergent subject matter, and separate search requirements.

